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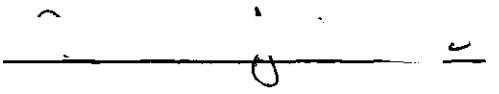

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EFFECTS ON GROWTH AND TOXIN PRODUCTION OF EXPOSURE OF SPORES OF THE CRAIG AND I-8G-F STRAINS OF CLOSTRIDIUM BOTULINUM TYPE F TO SUBLETHAL DOSES OF GAMMA IRRADIATION

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EFFECTS ON GROWTH AND TOXIN PRODUCTION OF EXPOSURE OF SPORES OF THE CRAIG AND I-8G-F STRAINS OF CLOSTRIDIUM BOTULINUM TYPE F TO SUBLETHAL DOSES OF GAMMA IRRADIATION

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I dedicate this thesis to my wife and children who have silently and without complaint weathered my unpredictable moods and whose help and encouragement have made whatever I have done possible.

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SUMMARY

The purpose of this research was to determine the effects on growth and toxin production, compared to unirradiated controls, of exposure of spores of the Craig and I-8G-F strains of Clostridium botulinum type F to sublethal doses of gamma irradiation.

Spores of the Craig and I-8G-F strains of Clostridium botulinum type F were subjected to 0.1 and 0.2 megarad doses of cesium-137 gamma irradiation. The effects of the irradiation of spores on growth and toxin production as compared to an unirradiated control culture were followed. Microscopic examinations and optical density readings were used to evaluate the effects of irradiation on vegetative growth, while mouse bioassay determinations were used to ascertain the effects of irradiation on toxin production.

Based on the research reported in this text, the data obtained on the post-irradiation growth response at 30 C of spores of Clostridium botulinum type F, strain I-8G-F subjected to 0.1 megarad and 0.2 megarad gamma irradiation are variable. However, toxin titers produced by the irradiated cultures at 30 C are consistently higher than those produced by an unirradiated control culture. No spore outgrowth or toxin production occurred in any of the I-8G-F cultures incubated at 10 C post-irradiation.

Cultures grown from spores of Clostridium botulinum type F, strain Craig irradiated with 0.1 megarad gamma irradiation produce higher optical density readings and cultures grown from spores subjected to 0.2 megarad

gamma irradiation attain lower optical density readings than unirradiated control cultures at 25 C. Toxin appears earlier and is consistently higher in the irradiated cultures than in the unirradiated control cultures at 25 C. At 10 C no spore outgrowth or toxin production by the Craig strain occurred in cultures inoculated with spores subjected to 0.2 megarad gamma irradiation. However, the 0.1 megarad irradiated culture of the Craig strain attained higher optical density readings and earlier toxin production than the unirradiated control culture.

CHAPTER I

INTRODUCTION

Radiation sterilization of food for human consumption is difficult because it requires a dose large enough to cause objectional organoleptic changes. Most radiation research pertaining to foods is therefore concentrated on studying the effects of "pasteurizing" doses of radiation (0.1-0.6 megarad) on the microbial and chemical components of food products. Pasteurizing doses of radiation are those that reduce the microbial population but do not destroy all organisms present. Several investigators have reported on the radiation resistance of bacterial spores and on the effects of sublethal doses of radiation on the toxicity produced by spores of Clostridium botulinum types A, B, C, D, and E. However, limited research has been done on the effects of sublethal doses of radiation on the spores of Clostridium botulinum type F. The purpose of this research was to study the effects of sublethal doses of gamma irradiation on the growth and toxin production of spores of the Craig and the I-8G-F strains of Clostridium botulinum type F.

Clostridium botulinum is a gram positive, anaerobic, sporeforming, rod-shaped organism. Its spores are widely distributed in nature, being found commonly in soil, mud, and the intestinal contents of animals. Thus, the opportunity for contaminating food with this organism is widespread.

Botulinum toxin is the most potent poison known to man. Although the toxin molecule is a protein, it is capable of passing into the lymphatic system from the upper intestinal tract. By means that are not yet understood, the toxin acts on certain neuromuscular junctions, interfering with the release of acetylcholine or combining with the acetycholine after its release, thus preventing the passage of nerve impulses. The muscles involved in respiration are particularly affected and death results from asphyxiation.

Botulism is a disease of man and other animals. Reports of human botulism have come mainly from North America, Europe, and Japan, although two outbreaks have been reported in Argentina and two in Australia (Meyer, 1956). The true incidence cannot be determined because of frequent failure to recognize the disease. In the United States there are usually no more than 10 or 12 verified outbreaks each year, with a total of 20-25 cases. For the past quarter century Germany has had 10 or 12 outbreaks (30 to 40 cases) annually (Meyer, 1956).

Since World War II the incidence in France has been similar to that in Germany (Meyer, 1956). In Japan, botulism was first recognized in 1951, but subsequently that country has averaged 4 outbreaks with about 8 cases per year (Nakamura, 1963). Between 1919 and 1954 Canada suffered a total of 14 known outbreaks, with an average of 4 cases each (Meyer, 1956). Although the incidence of botulism is low, the mortality rate is high. Almost two-thirds of the 1350 cases reported in the United States between 1899 and 1954 resulted in death (Meyer, 1956). In Europe the mortality rate is much lower, averaging 19 percent for the more than 4000 cases reported by Meyer (1956). The average mortality

rate in Japan has been 26 percent (Nakamura, 1963).

Frequently, gastric symptoms are the first indications of botulism, with nausea and vomiting appearing 12 to 18 hours after ingestion of the contaminated food. The patient may complain of a dry mouth during this time. Neurologic symptoms soon develop with resulting double vision, muscular weakness, and difficulty in talking and swallowing. Respiratory paralysis follows, death in fatal cases usually occurring in 3 to 6 days. In some cases, complete recovery may require several months.

The foods incriminated in outbreaks of botulism almost invariably are: (a) given an inadequate preliminary preparatory treatment such as heating, salting, smoking, brining, or pickling; (b) allowed to stand at a temperature that will permit the growth of Clostridium botulinum; and (c) eaten without cooking. In the United States, home canned vegetables are involved in the majority of cases (Meyer, 1950). Pork products are the major contributors in Europe, with salted or pickled fish also frequently involved (Meyer, 1956). A pickled relish called "izushi", made of raw fish, rice, and diced vegetables, has been the cause of over 90 percent of the outbreaks in Japan (Nakamura, 1963).

Six distinct types of Clostridium botulinum are now recognized. They are designated by the letters A, B, C, D, E, and F and are differentiated by the serological specificity of their toxins. Types A, B, and E have been responsible for all but a few of the known outbreaks among humans. Type F was first reported in 1960 and has been involved in two known outbreaks: one caused by home prepared liver paste in Denmark (Møller and Scheibel, 1960) and a second outbreak caused by home-made venison jerky in the United States (Morbidity Mortality Weekly

Rept., 1966). Types C and D have rarely been implicated in outbreaks of human botulism but they cause huge losses in wild and domestic animals.

Incidence of Type E Botulism

Clostridium botulinum type F, strain Craig, is a non-proteolytic organism similar in many characteristics to Clostridium botulinum type E. For this reason, the historical background of type E botulism has a bearing on the study of this organism.

The first reported outbreak of type E botulism occurred in Cooperstown, New York in 1932. The incident involved 3 persons and resulted in one death. The vehicle was smoked salmon, imported from Labrador (Hazen, 1938). A second outbreak occurred in 1934 in Westchester, New York resulting in 3 cases of human botulism and one death. In this instance, the incriminated food was commercially canned sprats from Germany (Hazen, 1937). In 1936, Gunnison, Cummings, and Meyer (1936) proposed the designation Clostridium botulinum type E for several cultures sent to them for identification by Russian bacteriologists. These organisms had been isolated from the intestines and muscles of sturgeon. In 1941, mushrooms from Yugoslavia, canned in California, were responsible for 3 cases of type E human botulism resulting in one death. This incident, reported by Geiger (1941), occurred in San Francisco.

In Nanaimo, B. C., the ingestion of home-canned salmon by a father, mother, and son resulted in the death of all three. Clostridium botulinum type E was identified as the causative agent (Dolman and Kerr, 1947). Two cases of type E botulism occurred in Canada in 1950 due to home-pickled herring. One of the men involved died (Dolman et al., 1950).

Several years later, in 1955, salmon eggs were responsible for still another outbreak of type E botulism in Canada. The outbreak involved 3 persons, one of whom died. The implicated foodstuff was salmon egg cheese made by traditional Indian tribal methods. A strain of Clostridium botulinum type E was isolated both from the stomach contents of the fatal case and from a sample of the salmon eggs consumed by the victims (Dolman, Darby, and Lane, 1955). Brocklehurst (1957) reported on a fatal outbreak of botulism among Labrador Eskimos. This type E outbreak was fatal to 6 of 8 of the Eskimos after they had eaten seal flipper that had been stored in a petroleum can.

Specialized foods as prepared by certain groups have been indicted in several type E botulism outbreaks. In Japan, since the disease was first recognized in 1951, 90 percent of the outbreaks have been caused by "izushi", which has already been described. Alaskan Eskimos like "muktuk", consisting of beluga whale flippers either dried or preserved in seal oil. The Indians of the northwest Pacific Coast favor salmon egg cheese. Some of the people of Norway eat salted trout or "rakefish" as a special dish. All of these have been implicated at one time in a botulism outbreak.

In 1960, plastic vacuum-packed smoked ciscoes caught in Lake Superior caused the deaths of 2 individuals in Minneapolis, Minnesota and in Forks, Washington, home canned salmon eggs caused 4 cases of type E botulism, resulting in 1 fatality (Eadie, 1964). In 1962, Bauman reported an outbreak of type E botulism related to the ingestion of preserved flounder in Russia (1962). It was in 1963 that the general American public became aware of Clostridium botulinum type E. In March, 3 women

ate a lunch of tuna fish salad and 2 of these women subsequently died (Johnston, et al., 1963). Type E botulism was diagnosed and the causative organism isolated from the empty tuna can. Other cans from the same lot were recovered from grocers' shelves; some of them also contained the organism. In late September and early October of 1963, two additional outbreaks of type E botulism occurred simultaneously; both were traced to the consumption of smoked fish from the Great Lakes (Morbidity Mortality Weekly Rept., 1963). A man and wife from Kalamazoo, Michigan, purchased a smoked whitefish while taking a motor trip through upper Michigan. Both contracted botulism and died. Clostridium botulinum type E was recovered from the remains of the fish. Immediately thereafter, botulism was diagnosed in several patients hospitalized in Nashville and in Knoxville, Tennessee. The outbreak eventually involved 17 patients (5 deaths) in the states of Tennessee, Alabama, and Kentucky. A single shipment of smoked whitefish chubs packed by a firm in Michigan was incriminated.

In all, there had been 76 outbreaks of type E botulism reported by October 25, 1963. Of these 44 had occurred in Japan, 7 in Alaska, 11 in the United States apart from Alaska, 11 in Canada, 3 in Sweden, 2 in Denmark, and 2 in the Soviet Union (Morbidity Mortality Weekly Rept., 1963).

In 1964 Kautter reported 3 cases of botulism from smoked ciscoes in which 2 of the 3 individuals died. The causative agent was Clostridium botulinum type E. In the same year four adults developed botulism from eating raw salmon eggs from a single one-quart jar (Ager and Dolman, 1964). One person died as a result of this outbreak. And 3 cases

(1 fatal) of food borne intoxication were reported that were caused by Clostridium botulinum type E in homemade gefilte fish prepared from fresh whitefish (Morbidity Mortality Weekly Rept., 1967).

The obvious association of type E botulism with fish products finally led investigators to study the occurrence of Clostridium botulinum spores in aquatic life and sediments. They found that foci of high concentration of Clostridium botulinum type E occurred in certain marine areas of the northern latitudes. Explanations of this distribution emphasized a terrestrial origin, with the organism being washed down from the surrounding land masses to the waters which serve as catchment basins (Dolman and Iida, 1963). Thus the presence of deposits of type E spores in lakes and in the soil of Hokkaido, Japan (Kanzawa, 1960; Nakamura, et al., 1963), Sweden (Johannsen, 1963), and British Columbia, Canada (Dolman and Iida, 1963) have been correlated with the frequent occurrence of type E in the northern Pacific Ocean and the Baltic Sea. Foster et al., (1965), using toxin neutralization tests, showed that Clostridium botulinum type E was present in 9 of 10 locations in Lake Michigan. The organism itself was found more frequently in the intestinal tracts of fish than on gills, liver, or external surfaces. In addition, over 75 percent of the cultures prepared from intestines of fish caught in one large bay in Lake Michigan contained type E toxin. Ward and Carroll (1965) demonstrated the presence of Clostridium botulinum type E in estuarine waters of the Gulf of Mexico. They isolated Clostridium botulinum type E from mud samples collected at various points in Galveston Bay, Texas. Other investigators made an intensive study of the intestinal contents of more than 3000 fish from Lakes Erie, Superior, Huron, and

Michigan which were examined for Clostridium botulinum type E (Bott et al., 1966). Incidence figures expressed as percent fish tested were: Lake Erie, 1 percent; Lake Superior, 1 percent; Lake Huron, 4 percent; the main body of Lake Michigan, 9 percent; and Green Bay (on Lake Michigan), 57 percent. They concluded that Clostridium botulinum type E appeared to be widely but unevenly distributed in the Great Lakes, and that fish from all areas were potential carriers. In a follow-up study in 1968, it was reported that the exceptionally high carrier rate in fish in Green Bay was correlated with the frequency with which the organism could be demonstrated in Green Bay sediments (Bott et al., 1968). The following reasons were given for the presence of Clostridium botulinum type E in samples: 1) After spores are washed from the soil into the bay, multiplication in the bay itself occurred. 2) Live fish and aquatic birds harboring the organism would be active in its dissemination and on their death would be foci for its multiplication.

Ward et al., (1967) demonstrated the same situation in a survey of the U.S. Gulf Coast for the presence of Clostridium botulinum. In sediment samples and animals collected during the warm weather months between Key West, Florida, and Brownsville, Texas, Clostridium botulinum type E was demonstrable. These investigators found that the incidence was somewhat higher in the eastern Gulf animals, but the organism was also present to the southernmost limits of both Texas and Florida. Similar studies were done on marine sediments and oysters (Crassostrea virginica) from Mobile Bay, Alabama (Presnell, Miescier, and Hill, 1967). Their findings showed Clostridium botulinum type E in both marine sediments and in oysters in Mobile Bay. Craig et al., (1968) investigated the

the incidence of Clostridium botulinum type E in salmon and other fish in the Pacific Northwest. The organism was detected by the identification of type E toxin in enrichment cultures of samples of salmon, sole, cod, oysters, clams, and Dungeness crabs from ocean waters along the coasts of Oregon and Washington.

Originally, there had been no regulations for the processing of smoked fish in the Great Lakes states. Each processor salted and smoked his product as he saw fit. There were no standards for salt, moisture, or heat treatment. However, following the type E botulism outbreaks of 1960 and 1963, the Food and Drug Administration issued a warning against the consumption and distribution of smoked fish from the Great Lakes area. Housewives were advised not to use smoked fish from the Great Lakes unless the product was known to have been either (1) heated to at least 180 F for 30 minutes after packaging and thereafter kept under refrigeration, or (2) frozen immediately after packaging and maintained continuously in a frozen condition.

This action promptly stimulated the issuance of processing regulations by several states and concerned municipal agencies. The states of Michigan, Wisconsin, Minnesota, and Illinois require that smoked fish be heated to an internal temperature of at least 180 F for 30 minutes during processing. The rules for control of temperature during distribution, and the permissible methods of packaging, vary considerably from state to state. Freezing as an alternative to the heating requirement is generally allowed, as originally suggested by the Food and Drug Administration. All the regulations mentioned were designed to protect the public from the hazard of botulism.

Incidence of Type F Botulism

The late identification of type F Clostridium botulinum has been attributed by Dolman and Murakami (1961) to its infrequent occurrence in nature, although recent publications indicate that the organism is not restricted to the locality of its original discovery. Clostridium botulinum type F was first isolated from liver paste that was associated with human botulism on the Danish island of Langeland (Møller and Scheibel, 1960). One person died; three others showed typical symptoms of the disease. The toxin of type F was demonstrated in cultures from samples of marine sediments taken off the coasts of Oregon and California (Eklund and Poysky, 1965). Then, during a survey of the natural distribution of Clostridium botulinum type E in the Pacific Northwest, Craig isolated and identified a pure culture of Clostridium botulinum type F in a sample taken from a sockeye salmon (Craig and Pilcher, 1966). Before that time, Clostridium botulinum type F had been reported only twice.

Type F botulism has been responsible for just one known outbreak of botulism in the United States. This occurrence was reported from California in 1966, when 3 persons contracted type F botulism after eating home-made venison jerky. All 3 patients survived (Morbidity Mortality Weekly Rept., 1966).

The toxin of Clostridium botulinum type F was identified in cultures of mud samples from Eastern North Dakota (Wentz et al., 1967). Type F toxin was also identified in a culture of a mixture of viscera and gills from a fish caught in the Atchafalaya River, Louisiana (Ward et al., 1967). However, neither group of investigators was able to

isolate the causative organism itself.

In the course of an investigation on the prevalence of Clostridium botulinum type E in crabs in the Middle Atlantic region of the United States, samples of either gills or viscera of two crabs (Callinectes sapidus) collected at the mouth of the York River channel yielded cultures of Clostridium botulinum type F (Williams-Walls, 1968). This was the first report on the isolation of a proteolytic Clostridium botulinum type F found in the United States, and the first demonstration of the natural occurrence of this immunologic type on the eastern coast of the United States.

The occurrence of Clostridium botulinum type F in aquatic habitats and its potential hazard in food products taken from such locations are the rationalefor the use of this particular immunologic type in the present study.

Irradiation Effects

The primary aim of food irradiation techniques is to extend the storage life of certain food items by subjecting them to low doses of ionizing radiation. In the food industry, radiation may be used for three purposes: (1) to prevent sprouting of rootcrops, such as potatoes; (2) to eliminate insects from grain before storage; and (3) to preserve food by inhibiting or destroying bacteria or other microorganisms. The amounts of radiation required for each of these effects differ greatly. A dose of 1000 to 4000 rads is highly effective as a sprout inhibitor when applied to onions or potatoes. Grains or cereals can be disinfected of insects at 20,000 to 50,000 rads, and at 50,000 rads it is possible

to sterilize the larvae of insects that lodge inside fruits. Pasteurization doses, generally in the range of 200,000 to 500,000 rads, will prolong the shelf life or storage time of certain foods. For example, the refrigerated storage life of fresh fish can be extended up to 30 days by such doses. Much higher doses, between 2 and 4.5 million rads, are required for "sterilization" of foods for long-time storage without refrigeration. Bacon and other pork products, chicken, and beef can be prepacked and irradiated at 4.5 million rads. These meats are reported to be well preserved (but not particularly palatable) after a year's storage at room temperature following the treatment (Casarett, 1968).

There are, however, certain problems associated with food sterilization by irradiation which have not been solved. Chemical changes may be produced in foods which can result in unpleasant flavors, loss of color, and changes in texture. The vitamin content of some foods may be decreased. Many of these changes are apparently associated with oxidation since the flavor and odor are much less affected if oxygen is removed from the system. Furthermore, many of these changes will disappear during postirradiation storage; the pleasant flavor may be restored and color return. It is possible that improved techniques of irradiation will minimize the problems mentioned (Casarett, 1968). There is also the question of whether toxic products are produced in certain foods during irradiation. Preliminary studies have shown that irradiated juices contain a material which can produce chromosomal aberrations in plants. However, exhaustive studies have demonstrated no toxic effects in mammals which were fed irradiated foods (Casarett, 1968).

A number of investigators have reported the effects of irradiation on growth, sporulation, and resistance of spores of clostridial species. Anellis and Koch (1962) reported on the radioresistance of 102 strains of types A and B Clostridium botulinum spores suspended in phosphate buffer. Type A strains showed higher radioresistance than those of type B although there was some overlapping. Schmidt et al., (1962) presented data on the radiation resistance of 6 strains of type E Clostridium botulinum in a beef stew substrate. By a comparison with 6 strains of type A and 5 strains of type B, he demonstrated that spores of type E exhibit only 45-55 percent of the resistance to ionizing radiation shown by spores of types A and B. He concluded that any radiation dose for food sterilization based upon the maximum resistance of type A and B strains would provide ample protection against type E strains.

The amount of radiation required to sterilize canned ground beef inoculated with type A and type B Clostridium botulinum spores varied directly with the logarithm of the number of spores per gram of meat. Concerning the toxicity of the cultures, it was shown that the toxin was present only when large spore inocula were used (greater than 10^6 spores/gram) and that the toxin probably came from the spores themselves. Neither the type A nor the type B subcultures from the irradiated meat showed evidence of growth, but toxin was demonstrable from a type A inoculum of 2,670,000 or more spores/gram (Kempe and Graikoski, 1962). This raised the question: Can irradiation increase the amount of toxin derived from spores? Recent studies suggest that conditions which increase permeability of the spore or degrade protein may enhance the

toxicity of botulinus toxin (Bonventre and Kempe, 1960).

In work with canned bacon, it was found that a dosage of 4.5 Mrad was more than adequate as a safe sterilization process even under experimental conditions of unrealistically high levels of contamination with Clostridium botulinum (6×10^5 spores/can). The data strongly indicated that the sterilizing dose could be lowered to a range of 2.0-4.5 Mrad (Anellis et al., 1965). In a later study, a corroboration of this data was obtained with canned cured ham. In addition, it was observed that cans of cured ham containing Clostridium botulinum spores, irradiated with certain sublethal doses (0.5 to 1.5 Mrad), clearly evidenced an acceleration in visible spoilage over unirradiated controls (Anellis et al., 1967). Cann et al., (1965) observed a similar phenomenon in toxin production in vacuum packed fish containing type E botulinal spores irradiated at 0.3 Mrad. Anellis et al., (1965) also found that radiation survival curves of Clostridium botulinum strain 33A exhibited an exponential reduction in numbers which accounted for most of the population, followed by a "tail" comprising a very small residual number of spores which resisted death in the range of 3.0 to 9.0 Mrad dose levels. He concluded that until more definitive data is obtained, radiation food sterilization will have to take into account the entire survival curve, including the radioresistant "tail". In a still later study, it was observed that when pork loin was inoculated with 10^6 type A or type B Clostridium botulinum spores per can, the minimal experimental sterilizing dose was between 2.5-3.0 Mrad (Anellis et al., 1969).

Abrahamsson et al., (1965) studied toxin production by Clostridium botulinum type E in vacuum-packed, irradiated fresh fish, in relation

to changes in microflora. They found that after irradiation at 0.6 Mrad the microflora consisted solely of micrococci. This change in microflora lent further support to the view that oxygen scavenging by aerobic bacteria favors growth of clostridia. These survivors (micrococci) were sensitive to antibiotics. Thus, the use of irradiation in combination with antibiotics might be effective in controlling spoilage flora. Also of interest, 0.3 and 0.6 Mrad inhibited toxin production in these packs at 20 C for 40 hours; on further storage, for 64 hours at 20 C, the samples irradiated at 0.3 Mrad became toxic.

Roberts and Ingram (1965) presented data from which survival curves after gamma irradiation could be constructed for a number of clostridial species. Irradiation at a temperature of 18-23 C produced curves comprised of a "shoulder" extending to about 0.25-0.35 Mrad, followed by an exponential kill over 6-8 log cycles. They suggested that pasteurizing doses of radiation would have to be accompanied with complementary processes in order to obtain a degree of safety and that the "shoulder" may almost totally nullify the practical effects of low doses of radiation.

Kanzanas and Emerson (1968) demonstrated that irradiation at 0.1 or 0.2 Mrad approximately doubled the shelf life of yellow perch fillets when they were stored at 1 C. Fernandez et al., (1969) presented evidence that although ground beef can be effectively sterilized at 4.5 Mrad gamma irradiation, existing spores may introduce a significant degree of toxicity which is not necessarily detectable immediately after irradiation but becomes evident after 2 to 4 weeks of incubation at 30 C.

Very little has been contributed to the literature on the effects

on growth and toxin production of exposure of spores of Clostridium botulinum type F to sublethal doses of gamma irradiation. Williams-Walls (1969) demonstrated with type F spores that maximal toxin titers were higher in irradiated than in unirradiated control cultures. These data coincide with those reported by Kempe and Graikoski (1962) and observed by Cann et al., (1965) in their work with vacuum-packed fish. This research thesis was designed to obtain additional data on the effects of sublethal doses of gamma irradiation on the growth and toxin production of both a proteolytic and a non-proteolytic strain of Clostridium botulinum type F.

CHAPTER II

MATERIALS AND METHODS

Organisms Used

Both the Craig and the I-8G-F strains of Clostridium botulinum type F were obtained from laboratory stock cultures. Originally the Craig strain was received from the Aberdeen Culture Collection, Aberdeen, Scotland. The I-8G-F strain was isolated from a crab sample by Dr. Nancy W. Walls in her laboratory at the Engineering Experiment Station, Georgia Institute of Technology.

Production of Spores

I-8G-F Strain

Spores of the I-8G-F strain were produced in Type C Toxin Medium (see Appendix A) adjusted to pH 7.6 with 10N NaOH prior to autoclaving. One percent dextrose was added as a 20 percent sterile solution after the medium had been autoclaved. Starting with a frozen stock culture, a 10 percent inoculation was made into cooked meat medium (Difco-see Appendix A), at intervals of three days, for two successive transfers. The cultures were incubated at 30 C. After the second incubation period, a 10 percent inoculum was transferred into the Type C Toxin Medium, and the resulting culture incubated at 30 C for 12 days before the spores were harvested.

Craig Strain

One milliliter of spore stock of the Craig strain was inoculated

into 30 ml of cooked meat medium. This culture medium was incubated at 25 C for 3 days. A biphasic medium for spore production of the Craig strain was prepared as follows: 1200 ml of Trypticase Soy Agar (BBL-see Appendix A) was prepared in a 3200 ml modified Fernbach flask, autoclaved for 15 minutes at 15 lbs pressure, and allowed to solidify. The gelled agar was overlaid with 500 ml of sterile Trypticase Soy Broth (BBL-see Appendix A). The liquid portion of the 30 ml cooked meat culture was aseptically withdrawn from the meat particles, and inoculated into the broth phase of the biphasic medium. This culture was incubated at 25 C for three days before spores were harvested.

Harvesting of Spores

Six sterile 250 ml centrifuge bottles were filled with approximately 200 ml each of medium containing cells and spores. Sealed centrifuge cups, containing the bottles, were then spun at 4 C for 30 minutes at 2000 rpm (1000 x g) in an International refrigerated centrifuge.* The liquid portion was decanted, the spores resuspended in cold sterile deionized water, and pooled in a small screw cap Erlenmeyer flask. The spinning and collecting procedure was repeated until all the original media had been spun. The pooled spores and cells were then distributed among four bottles, and suspended in cold sterile deionized water, 200 ml per bottle. This spinning and washing procedure was repeated three times, until all the spores and cells were collected and spun in one bottle. The pellet was then transferred to a sterile 50 ml screw cap Erlenmeyer flask containing glass beads, suspended in a small amount of sterile deionized water, and the resulting spore stock refrigerated.

*IEC Model PR-2; International Equipment Co., Needham Hts., Mass.

Spore Cleaning Procedure

Whenever an experiment was initiated with an aliquot of the spore suspension it was necessary to remove all vegetative cells and cellular debris from the sample. The cleaning procedure is a modification of Grecz's method (Grecz et al., 1962) developed by Mr. G. R. Bell (Georgia Institute of Technology, Engineering Experiment Station). The procedure alternates sonication with digestion by trypsin and lysozyme at 45 C. Stock solutions were prepared as follows:

Trypsin (1:250) Difco - to contain 5 mg/ml solution

Lysozyme (6000 to 10,000 units/mg*) - to contain 10 mg/ml solution

These were filter-sterilized and kept under refrigeration. Into a sterile 150 ml beaker were placed: 2.0 ml spore stock, 1.0 ml trypsin solution, 1.0 ml lysozyme solution, and 46 ml cold sterile deionized water. The mixture was alternately subjected to ultrasonic oscillation at 20 KC with a Bronwill Biosonik III** probe at a setting of 67, and incubation in a water bath at 45 C, as follows:

1. Sonic treatment---------- 5 minutes
2. Incubation----------20 minutes
3. Sonic treatment---------- 5 minutes
4. Incubation----------30 minutes
5. Sonic treatment---------- 5 minutes
6. Incubation---------- 1 hour
7. Sonic treatment---------- 5 minutes

During sonic disruption sterile foil covered the beaker and the probe

* Nutritional Biochemical Corp., Cleveland, Ohio.
** Bronwill Scientific, Rochester, New York.

was inserted through an opening in the foil. After completion of the last sonic oscillation treatment, the mixture was spun down at 4 C at 15,000 rpm (25,000 x g) for 5 minutes in an International refrigerated centrifuge*. The supernatant liquid was decanted and the spores washed in cold sterile deionized water and spun down successively three times. To rid the spore suspension of vegetative cells, an equal portion of 95 percent ethanol was added and the spore suspension allowed to sit at room temperature for one hour, with occasional agitation. Again, the spores were washed three times with cold sterile deionized water, spinning after each washing at 15,000 rpm (25,000 x g) for 5 minutes. The spores were then resuspended in as little cold sterile deionized water as possible and stored in a sterile container with glass beads, at 4 C.

Irradiation Procedure

A cleaned spore stock was diluted 1:10 with sterile Sorenson's phosphate buffer (see Appendix A) at pH 7.0. Ten ml of the diluted spore suspension was placed in each of two 15 x 125 mm screw cap culture tubes. The tubes were kept immersed in a water and ice mixture at 0 C before, during, and after irradiation until inoculated into the medium chosen for the experiment. The tubes containing the spore suspension were subjected to 0.0, 0.1, and 0.2 Mrad irradiation in the center well of a cesium source having a uniform radiation field**. The cesium-137 gamma radiation source at the Georgia Institute of Technology was used. The samples were equidistant from the cesium-137 rods and this assured

* Model B-20; International Equipment Corp., Needham Heights, Massachusetts.
**The radiation source is a 12,000c cesium-137 source located at Georgia Tech in the Emerson Building.

equivalent radiation dosage to all samples. The average dose rate was 1.1 Mrad per hour as measured by both ferrous ion oxidation and Dupont MSC-300 light blue cellophane dosimeters.

Growth Medium

The growth medium used after irradiation was freshly prepared sterile Trypticase Soy Broth (see Appendix A) contained in 300 ml Nephelo culture flasks (Bellco). When it had cooled after autoclaving, to 244 ml of medium in each flask was added 6 ml of filter-sterile 20 percent ribose. Prior to inoculation, the medium was equilibrated for two hours at 10 C, 25 C, or 30 C. A 1 percent (by volume) inoculum of spore suspension was then added to duplicate flasks for each radiation level (0.0, 0.1, or 0.2 Mrad). The purpose was to achieve a final spore concentration in the growth medium that would be uniform for the unirradiated and the irradiated samples. The effect of irradiation on spore outgrowth and toxin production at two temperatures (10 C and 30 C for I-8G-F; 10 C and 25 C for Craig) was then studied.

Growth Measurements

Bacterial growth was determined by measuring turbidity at a wavelength of 600 nm on a Bausch and Lomb "Spectronic 20" colorimeter*. The colorimeter was adjusted to indicate zero optical density with a sterile blank of the growth medium. The determination of bacterial growth by this method gave a relative value rather than an absolute quantitation of cells per unit volume. Growth was also followed microscopically by means of stained smears (see Appendix A) to determine the point at which

*Bausch & Lomb, Inc., Rochester, New York.

the spores ceased taking up malachite green dye and became permeable to methylene blue dye, and to correlate the morphological state of cells with different optical density readings.

Toxin Titration

Toxin was assayed by the standard mouse bioassay procedure using 13 to 18 g, male or female, ICR strain white mice injected intraperitoneally with 0.5 ml of centrifuged culture supernatant fluid serially diluted in pH 7.0 gelatin diluent (see Appendix A). Toxin levels were estimated by utilizing two mice per dilution and expressed as LD_{50}'s.

Trypsinization Procedure

Both trypsin activated and untrypsinized culture supernatants were tested for toxicity. A culture supernatant fraction was adjusted to pH 6.0 and then trypsinized by incubating 1.6 ml of the supernatant fraction with 0.4 ml of a 5 percent solution of Difco 1:250 trypsin at pH 6.0 in a 37 C water bath for one hour (Duff et al., 1956).

Spore Titration

The parameter for viability of spores after irradiation was their ability to form colonies. The spore concentration of the I-8G-F strain was determined by serially diluting the spore suspension in 0.1 percent peptone water (see Appendix A) and inoculating triplicate samples of appropriate dilutions into 25 ml volumes of freshly prepared pork infusion medium (see Appendix A) in modified agar slant tubes. These were rotated between the hands to insure uniform mixing, rapidly cooled in an ice-water bath, and sealed with a plug containing 2 percent agar

(Difco) and 0.1 percent sodium thioglycollate (Difco). The tubes were incubated at 30 C for 7 days. Spore concentration was determined by counting those tubes containing between 10-200 colonies and then multiplying by the dilution factor.

The viability of Craig spores after irradiation was determined in the same manner as elucidated above except that Schmidt's counting medium (see Appendix A) was employed in place of the pork infusion medium. The tubes were incubated at 25 C for 36-48 hours before counting.

CHAPTER III

RESULTS

I. Proteolytic *Clostridium botulinum* Type F (Strain I-8G-F): Growth and Toxin Production after Irradiation at 0.0, 0.1, or 0.2 Megarad.

A. Growth and Toxin Production at 30 C.

Figures 1, 2, and 3 show the results of experiment number one on growth response and toxin production in trypticase soy broth at 30 C of spores irradiated with 0.0, 0.1, or 0.2 megarad of cesium 137 gamma irradiation. Approximately 12 hours after inoculation into trypticase soy broth, the spores of all three cultures showed signs of germination. This was determined by microscopic examination of spore stains which showed that the spores gradually lost the ability to take up malachite green and began to take up methylene blue stain around their edges as seen through the oil immersion lens. An occasional vegetative cell was also observed at this time. As shown in Table 1, outgrowth became evident by increased optical density readings at 13 hr for the unirradiated and 0.1 megarad irradiated cultures and at 15 hr for the 0.2 megarad irradiated culture.

At the end of the lag phase, all three cultures appeared to enter a normal logarithmic growth phase. The logarithmic growth phase in the unirradiated and 0.1 megarad irradiated cultures continued for the next 19 hr and both cultures reached the stationary growth phase 31 hr after inoculation. This stationary growth phase lasted 5 hr in both cultures; both began to autolyze after 36 hr of incubation. In the 0.2 megarad

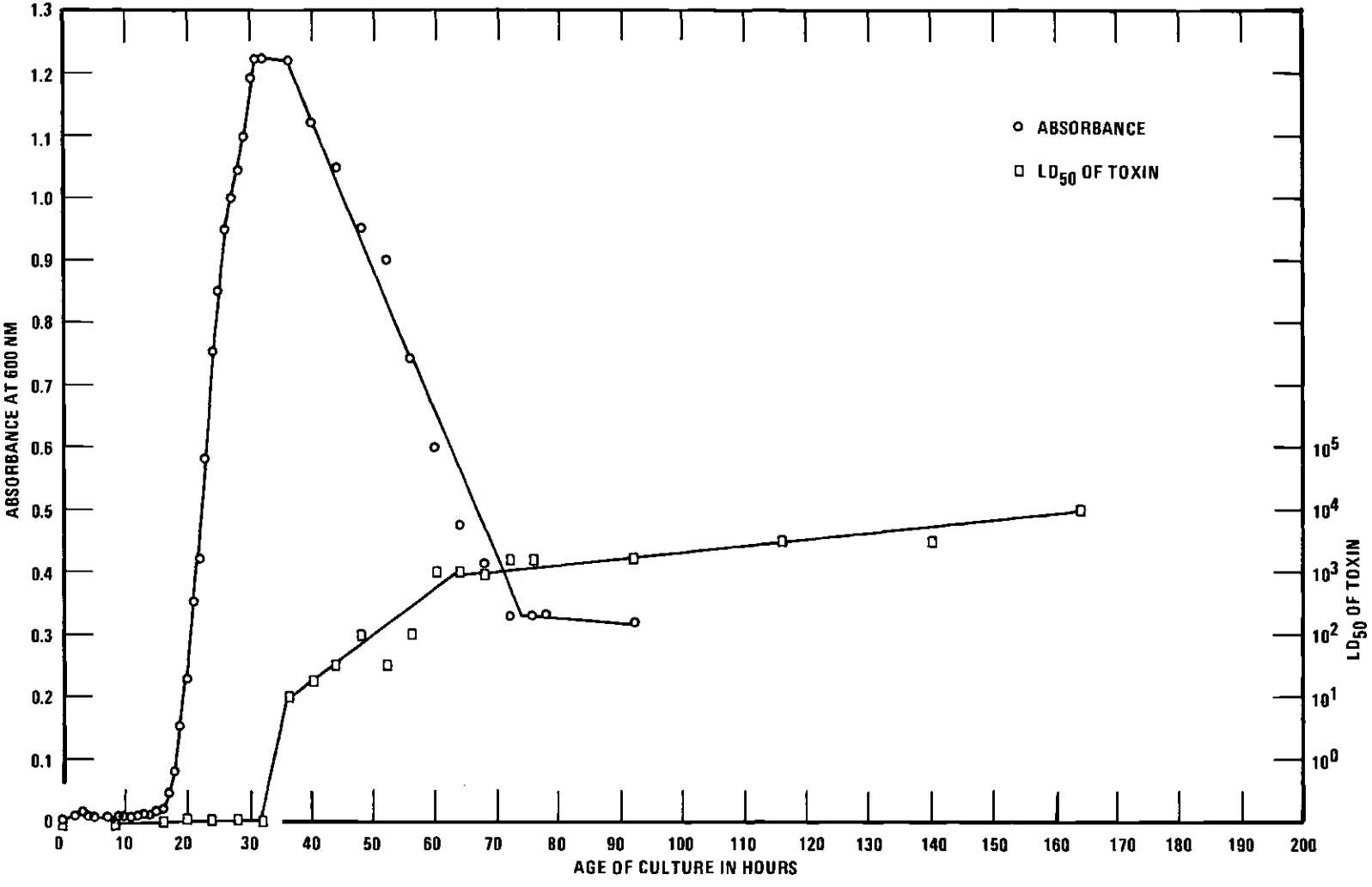

Figure 1. Growth and Toxin Production of Unirradiated Spores of Clostridium botulinum Type F, Strain I-8G-F in Trypticase Soy Broth at 30 C (Experiment 1).

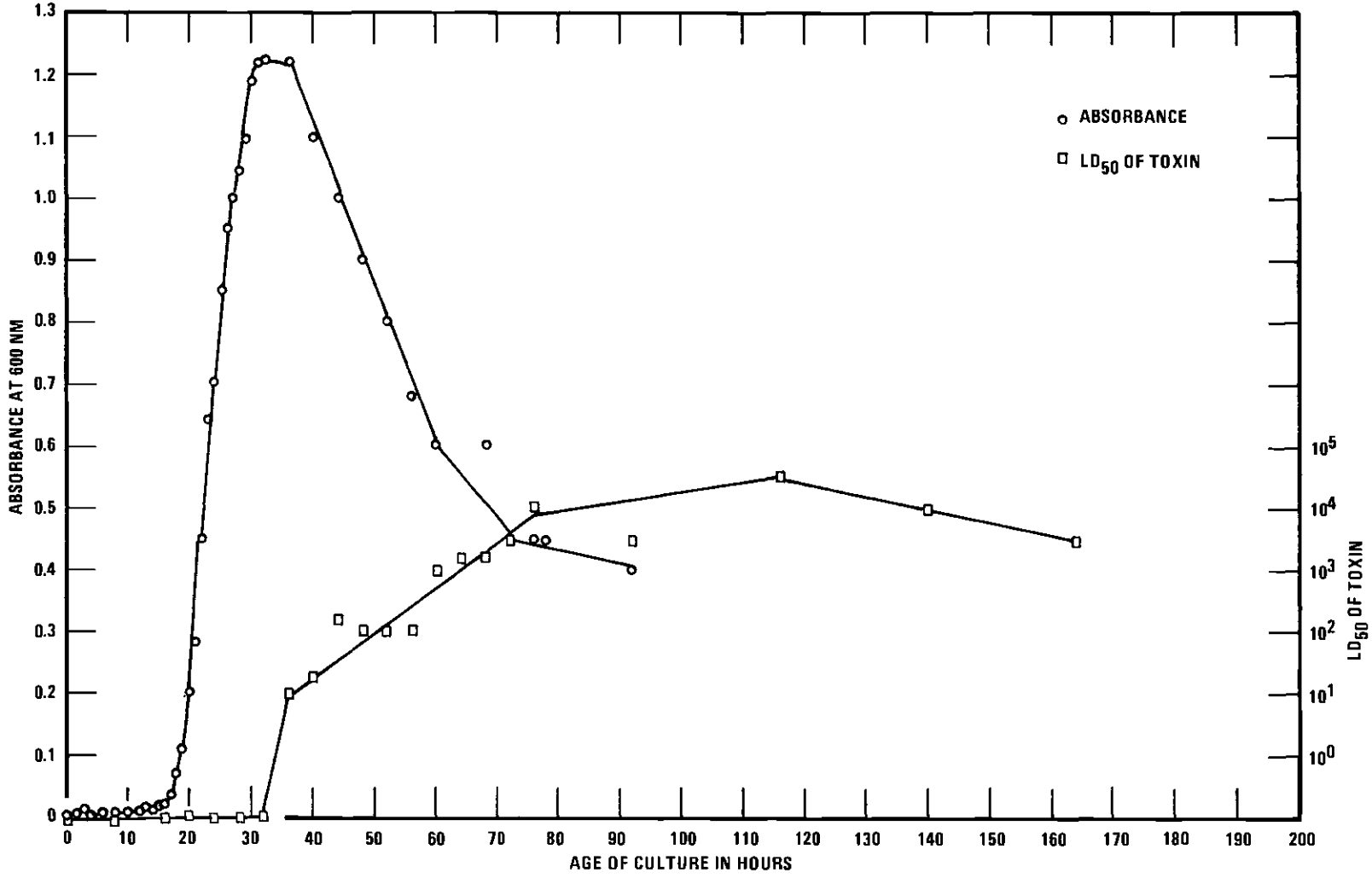

Figure 2. Effect of 0.1 Megarad of Cesium-137 Gamma Irradiation on Growth and Toxin Production of Spores of Clostridium botulinum Type F, Strain I-8G-F in Trypticase Soy Broth at 30 C (Experiment 1).

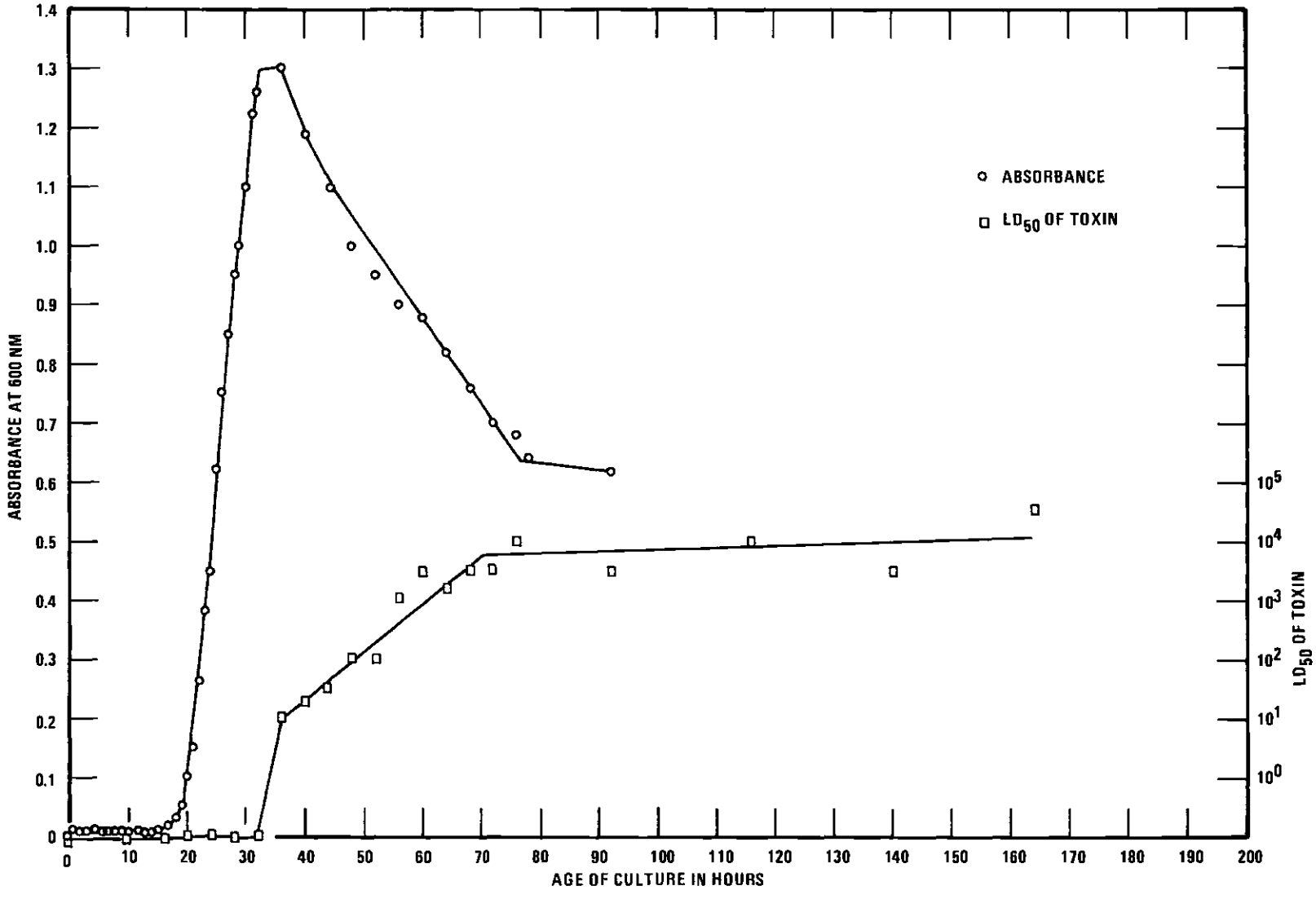

Figure 3. Effect of 0.2 Megarad of Cesium-137 Gamma Irradiation on Growth and Toxin Production of Spores of Clostridium botulinum Type F, Strain I-8G-F in Trypticase Soy Broth at 30 C (Experiment 1).

Table 1. Summary of Germination Times for 0.0, 0.1, and 0.2 Megarad Doses of Gamma Irradiation in Experiments on Clostridium botulinum Type F, Strain I-8G-F Incubated at 30 C.

Experiment Number	Germination Time in Hours		
	0.0 Megarad	0.1 Megarad	0.2 Megarad
One	13	13	13
Two	16	16	19
Three	16	15	14

irradiated culture, the logarithmic growth phase continued for 18 hr, the culture reaching stationary growth phase after 32 hr of incubation. Its stationary growth phase lasted only 4 hr so that this culture also began autolyzing after 36 hr incubation. Table 2 shows that the maximal optical density reached was somewhat less in the unirradiated and 0.1 megarad irradiated cultures than in the 0.2 megarad irradiated culture.

In all three cultures measurable toxin first appeared in the culture supernates at 36 hr; i.e., at the beginning of autolysis. After initial appearance, toxin titers were identical through the 40th hr of incubation for all three cultures. At 44 hr the 0.1 megarad irradiated culture exhibited a higher toxin titer than the unirradiated and the 0.2 megarad irradiated cultures. At 48 hr toxin titers were again identical in all three cultures. From 52 hr through 164 hr, either one or both of the irradiated cultures exhibited elevated toxin titers over that of the unirradiated control culture (Figures 1, 2, and 3).

Figures 4, 5, and 6 show the results obtained in another experiment (experiment number 3) on growth response and toxin production at 30 C of the I-8G-F strain. These data corroborate the data obtained in the first experiment. Spores of all three cultures showed signs of germination about 12 hr after inoculation into TSB. Outgrowth was detected colorimetrically at 14 hours for the 0.2 megarad irradiated culture, at 15 hours for the 0.1 megarad irradiated culture, and at 16 hours for the unirradiated control culture (see Table 1). All three cultures then appeared to enter a normal logarithmic growth phase. The logarithmic growth phase continued for 17 hours in the 0.2 megarad

Table 2. Maximum Absorbance and LD_{50} Obtained in Experiments on *Clostridium botulinum* Type F, Strain I-8G-F Incubated at 30 C.

Radiation Dosage	Experiment Number	Maximum OD Obtained	Age of Culture (Hrs.)	Initial Appearance of Toxin (Hrs.)	Maximum LD_{50} Toxin ($\times 10^{-4}$)	Age of Culture (Hrs.)
0.0 Megarad	1.	1.22	31	36	1.00	164
	2.	2.00	44	28	1.00	64
	3.	1.30	41	24	0.10	44
0.1 Megarad	1.	1.22	31	36	5.00	116
	2.	2.00	44	28	5.00	68
	3.	1.40	36	24	0.20	64
0.2 Megarad	1.	1.30	32	36	5.00	164
	2.	1.50	52	28	10.00	72
	3.	1.40	34	28	0.20	64

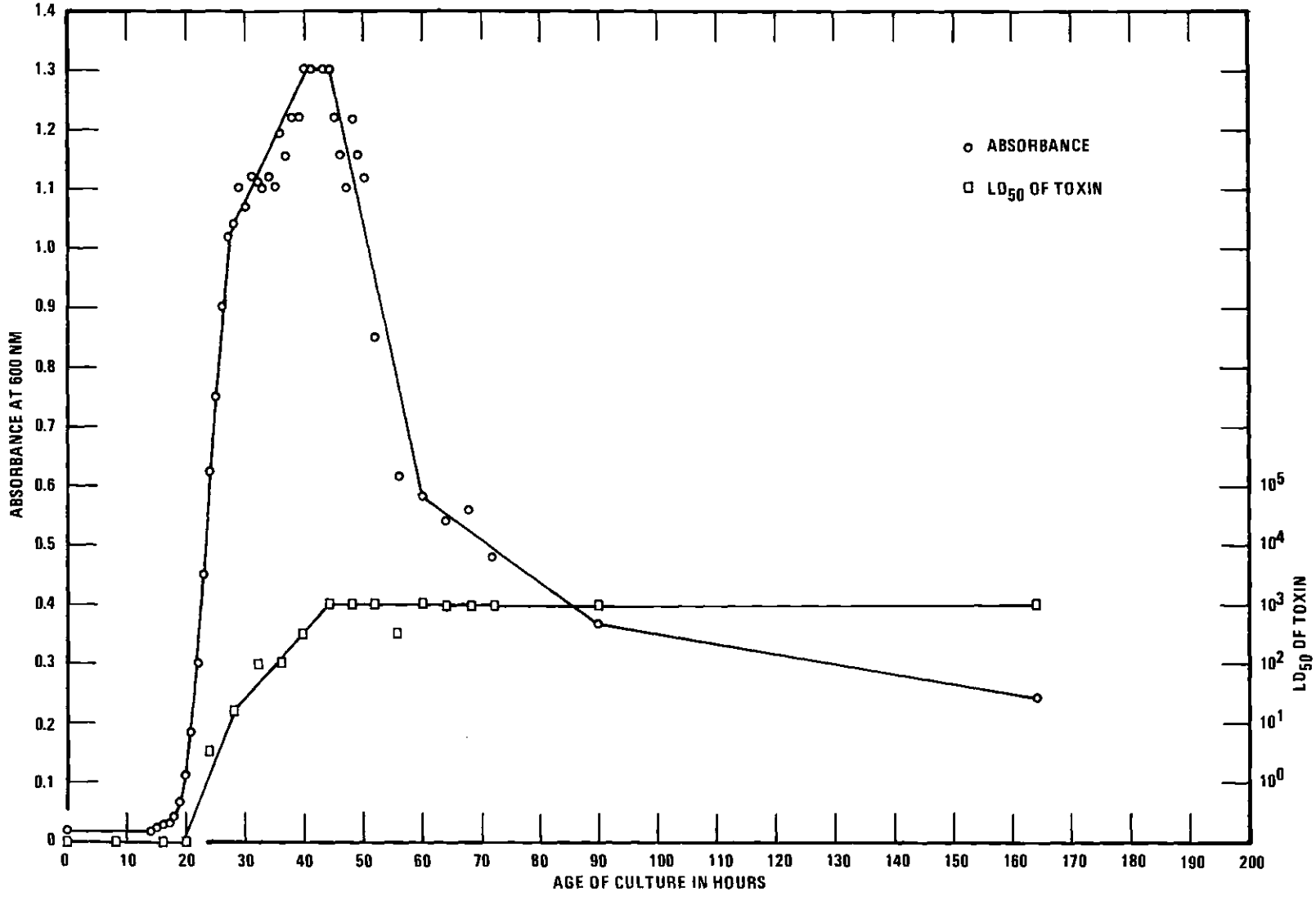

Figure 4. Growth and Toxin Production of Unirradiated Spores of Clostridium botulinum Type F, Strain I-8G-F in Trypticase Soy Broth at 30 C (Experiment 3).

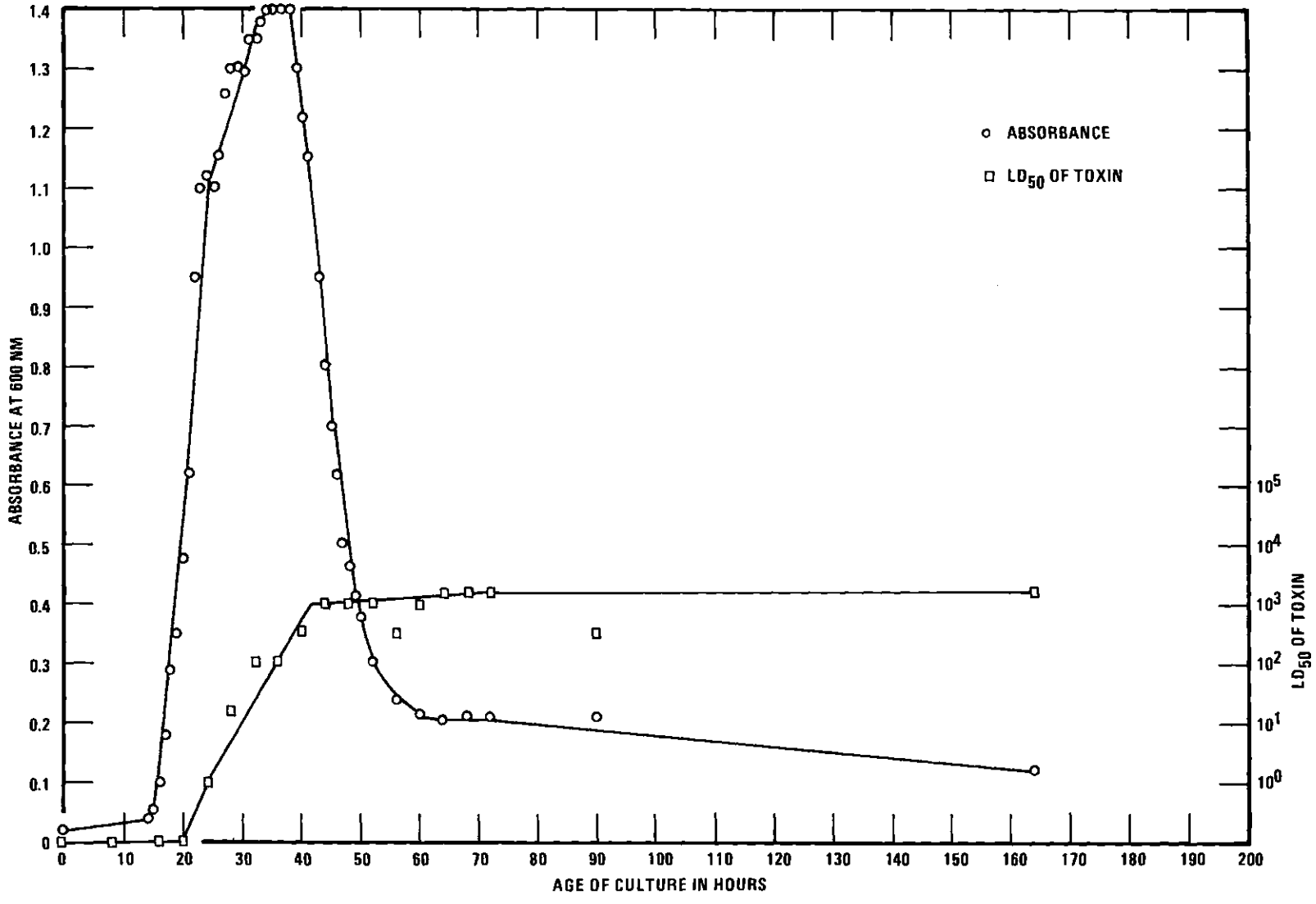

Figure 5. Effect of 0.1 Megarad of Cesium-137 Gamma Irradiation on Growth and Toxin Production of Spores of Clostridium botulinum Type F, Strain I-8G-F in Trypticase Soy Broth at 30 C (Experiment 3).

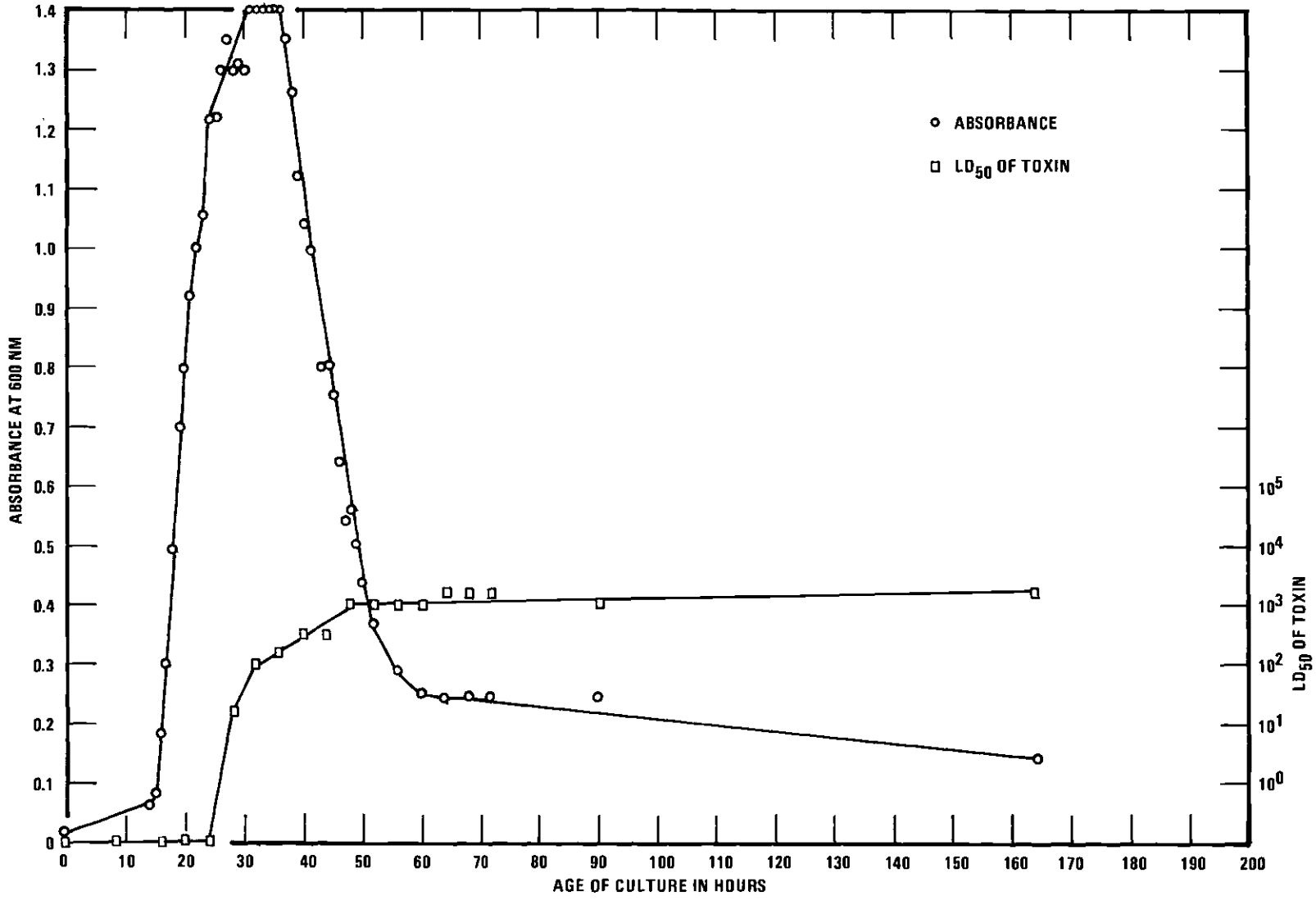

Figure 6. Effect of 0.2 Megarad of Cesium-137 Gamma Irradiation on Growth and Toxin Production of Spores of Clostridium botulinum Type F, Strain I-8G-F in Trypticase Soy Broth at 30 C (Experiment 3).

irradiated culture, 19 hours in the 0.1 megarad irradiated culture, and 24 hours in the unirradiated control culture, reaching stationary growth phase after 31, 34, and 40 hours incubation, respectively. The stationary growth phase lasted 4 hours for the unirradiated and the 0.1 megarad irradiated cultures with autolysis beginning at 44 hours and 38 hours, respectively. The 0.2 megarad irradiated culture remained in stationary growth phase for 5 hours with autolysis beginning at 36 hours. Table 2 shows that the maximal optical density reached was somewhat less in the unirradiated control culture than in the unirradiated cultures.

Toxin was first detected at 24 hr for both the unirradiated control culture and the 0.1 megarad irradiated culture and at 28 hr for the 0.2 megarad irradiated culture. Slightly elevated toxin titers were exhibited in the irradiated cultures over those of the unirradiated control cultures after 36, 40, 56, 64, 68, 72, and 164 hr incubation (Figures 4, 5, and 6). Toxin titer determinations at the other sampling points yielded equal toxin titers in all three cultures.

Figures 7, 8, and 9 show the results of a third replicate of this experiment (experiment number 2) on the growth response and toxin production at 30 C of irradiated cultures and an unirradiated control culture of Clostridium botulinum type F, strain I-8G-F. These data illustrate a phenomenon not observed in the other two experiments with the I-8G-F strain. The unirradiated and 0.1 megarad irradiated cultures entered logarithmic growth phase 16 hr after inoculation, the 0.2 megarad culture at 19 hr (see Table 1). In contrast to the response seen in the other experiments, however, 9 to 12 hr after entering the logarithmic growth phase all three cultures underwent partial lysis. An additional

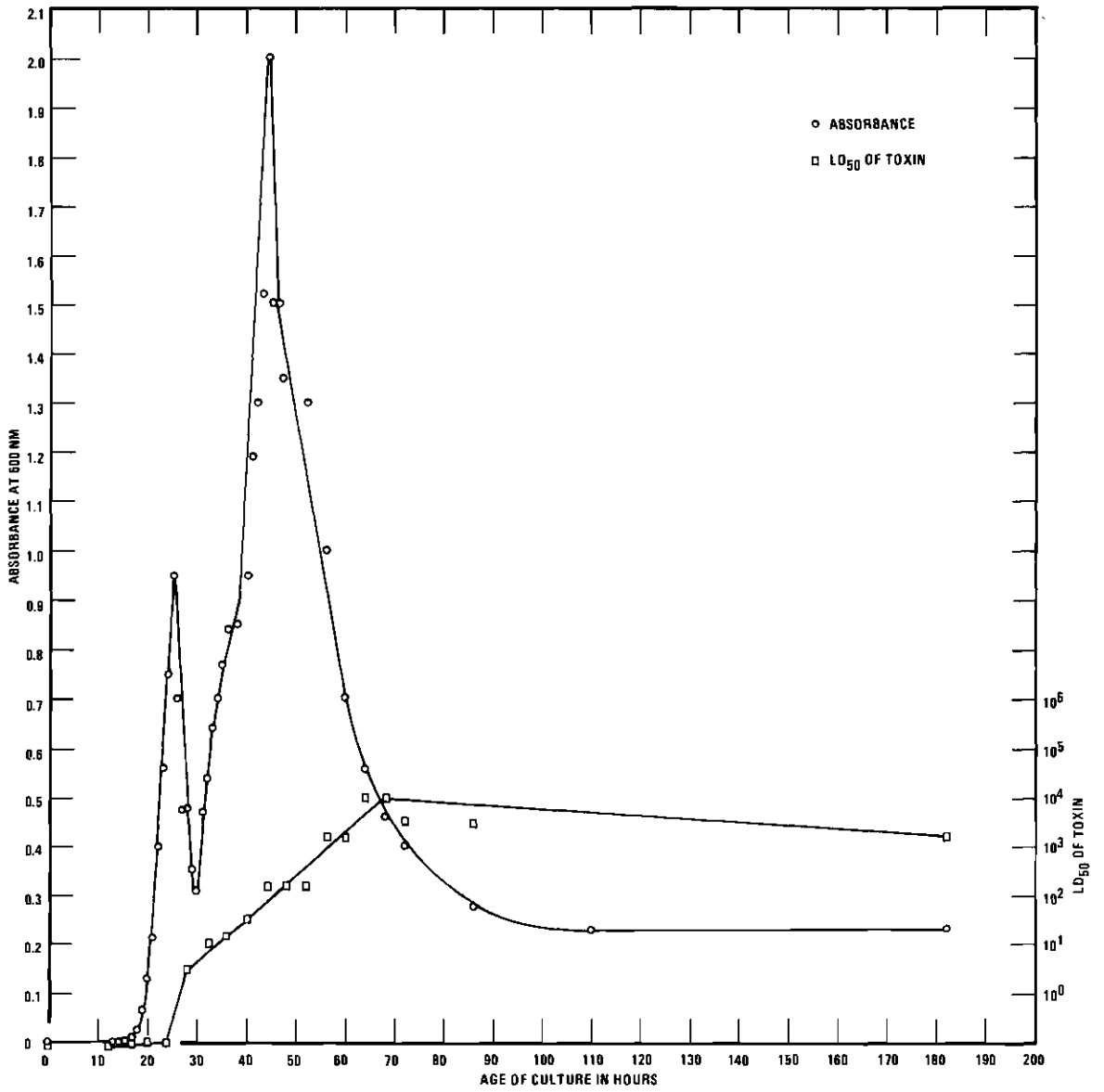

Figure 7. Growth and Toxin Production of Unirradiated Spores of Clostridium botulinum Type F, Strain I-8G-F in Trypticase Soy Broth at 30 C (Experiment 2).

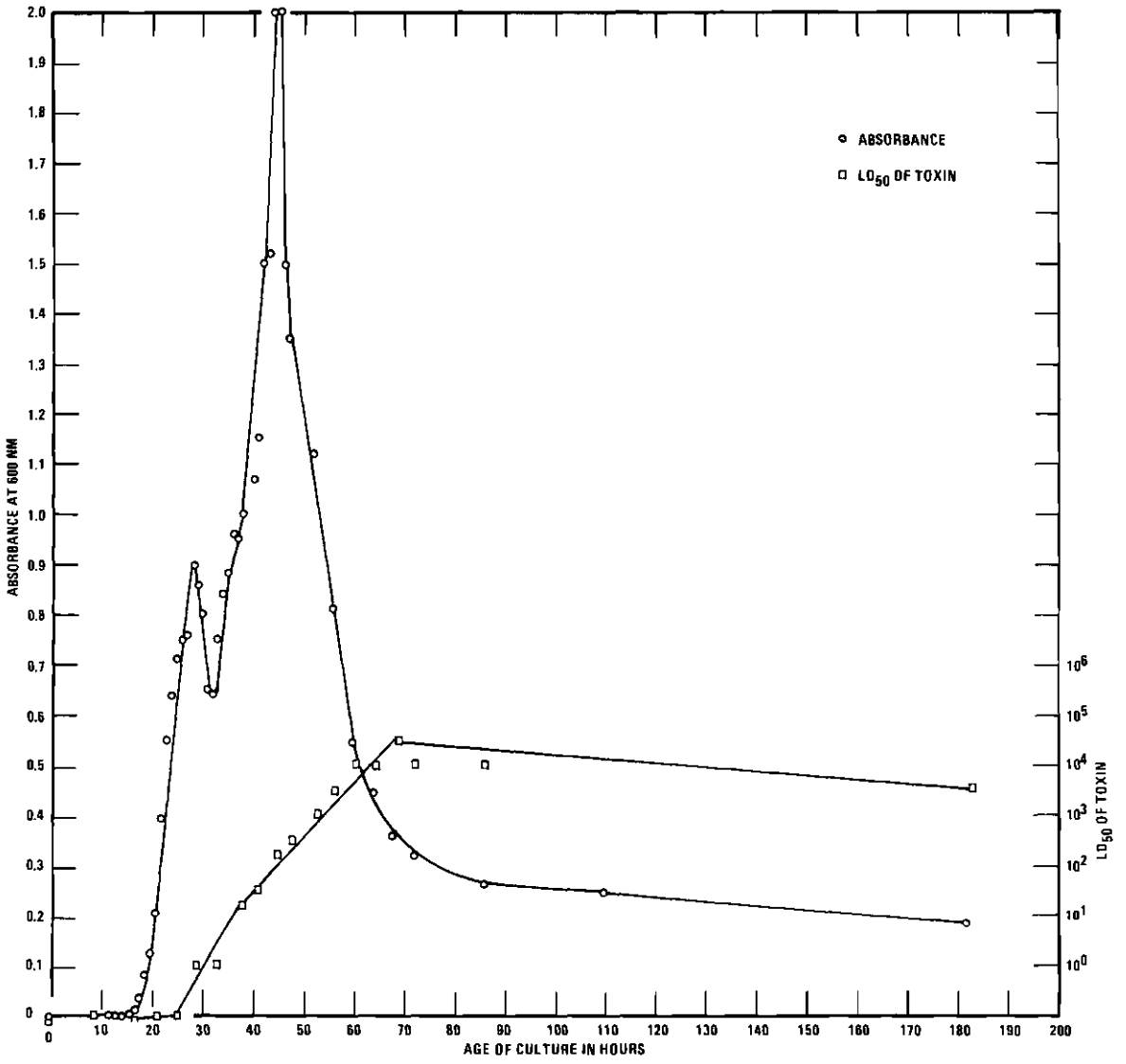

Figure 8. Effect of 0.1 Megarad of Cesium-137 Gamma Irradiation on Growth and Toxin Production of Spores of Clostridium botulinum Type F, Strain I-8G-F in Trypticase Soy Broth at 30 C (Experiment 2).

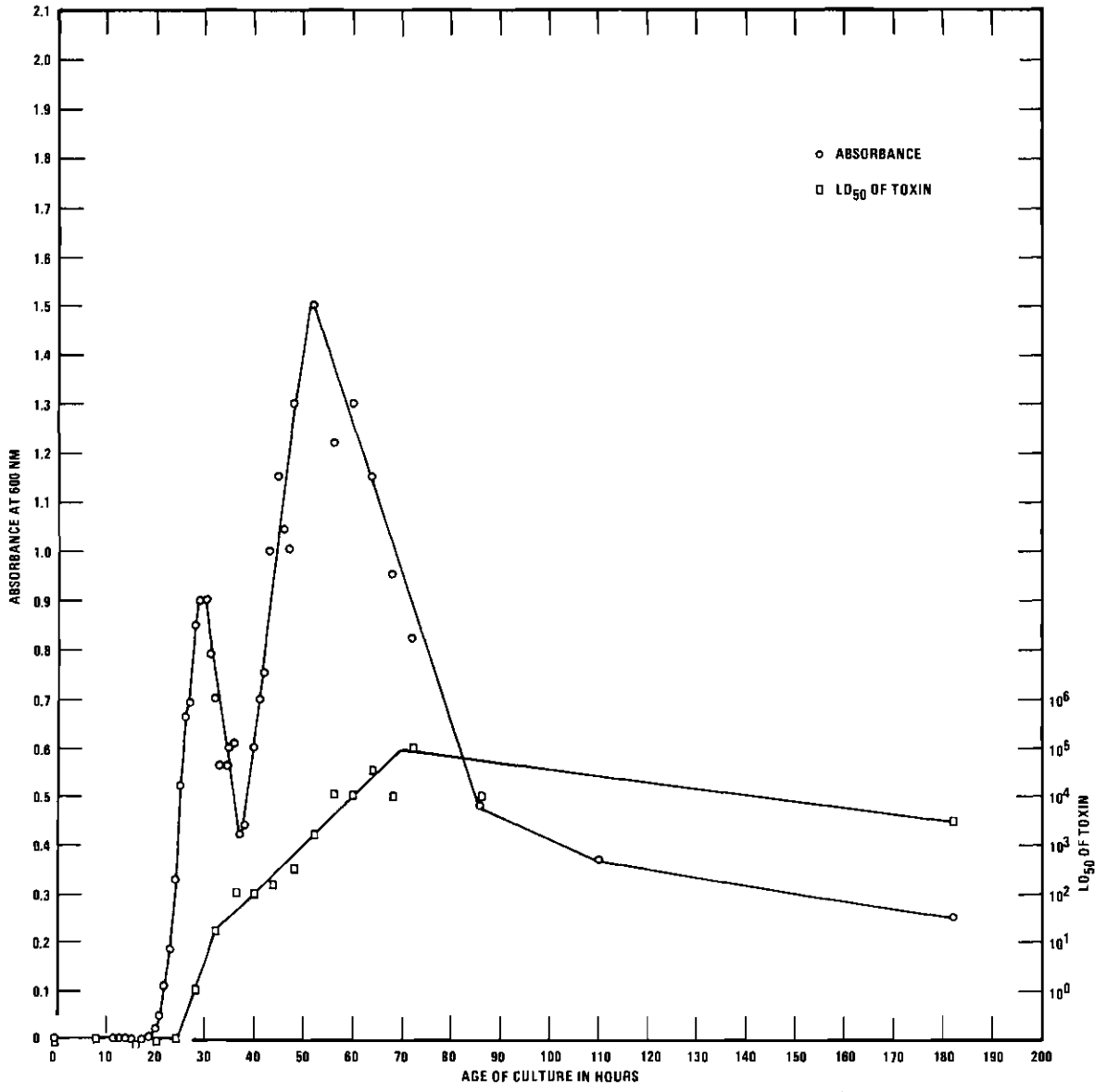

Figure 9. Effect of 0.2 Megarad of Cesium-137 Gamma Irradiation on Growth and Toxin Production of Spores of Clostridium botulinum Type F, Strain I-8G-F in Trypticase Soy Broth at 30 C (Experiment 2).

7 to 14 hours were required for the three cultures to reach the same optical densities observed before the lytic phenomenon occurred. The maximal optical densities reached in the unirradiated and 0.1 megarad irradiated cultures were higher than that reached by the 0.2 megarad irradiated culture (see Table 2). Autolysis occurred at 44 hr for the unirradiated control, at 45 hr for the 0.1 megarad irradiated culture, and at 52 hr for the 0.2 megarad irradiated culture.

Toxin was detected in all three cultures 28 hr after inoculation into the growth medium. Maximal toxin titers were attained after 64, 68, and 72 hr incubation for the unirradiated, 0.1 megarad irradiated culture, and 0.2 megarad irradiated culture, respectively (see Table 2). The toxin titers were consistently higher in the irradiated cultures than in the unirradiated control culture and the elevated toxin titers persisted in the irradiated cultures over a 182 hr period.

B. Growth and Toxin Production at 10 C.

Cultures of Clostridium botulinum type F, strain I-8G-F, incubated at 10 C after irradiation and observed for growth response and toxin production, gave negative results. No growth of the unirradiated control culture or the irradiated cultures was detected colorimetrically during incubation at 10 C for 30 days and no toxin was demonstrable in the 10 C cultures with or without trypsin activation.

II. Non-Proteolytic Clostridium botulinum Type F (Strain Craig): Growth and Toxin Production after Irradiation at 0.0, 0.1, or 0.2 Megarad.

A. Growth and Toxin Production at 25 C.

Figures 10, 11, and 12 show the results obtained (experiment number 4) on growth response and toxin production in trypticase soy

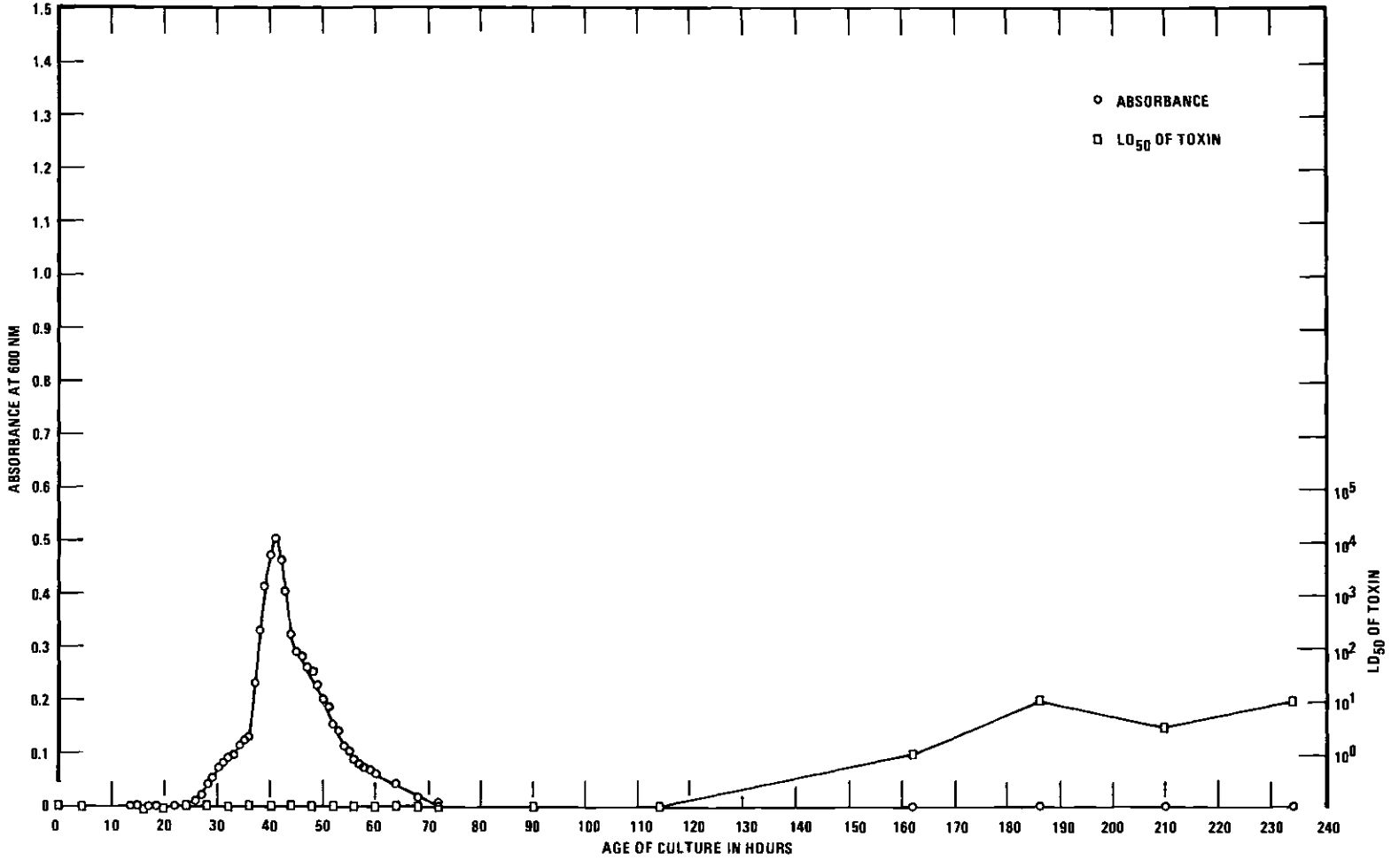

Figure 10. Growth and Toxin Production of Unirradiated Spores of Clostridium botulinum Type F, Strain Craig in Trypticase Soy Broth at 25 C (Experiment 4).

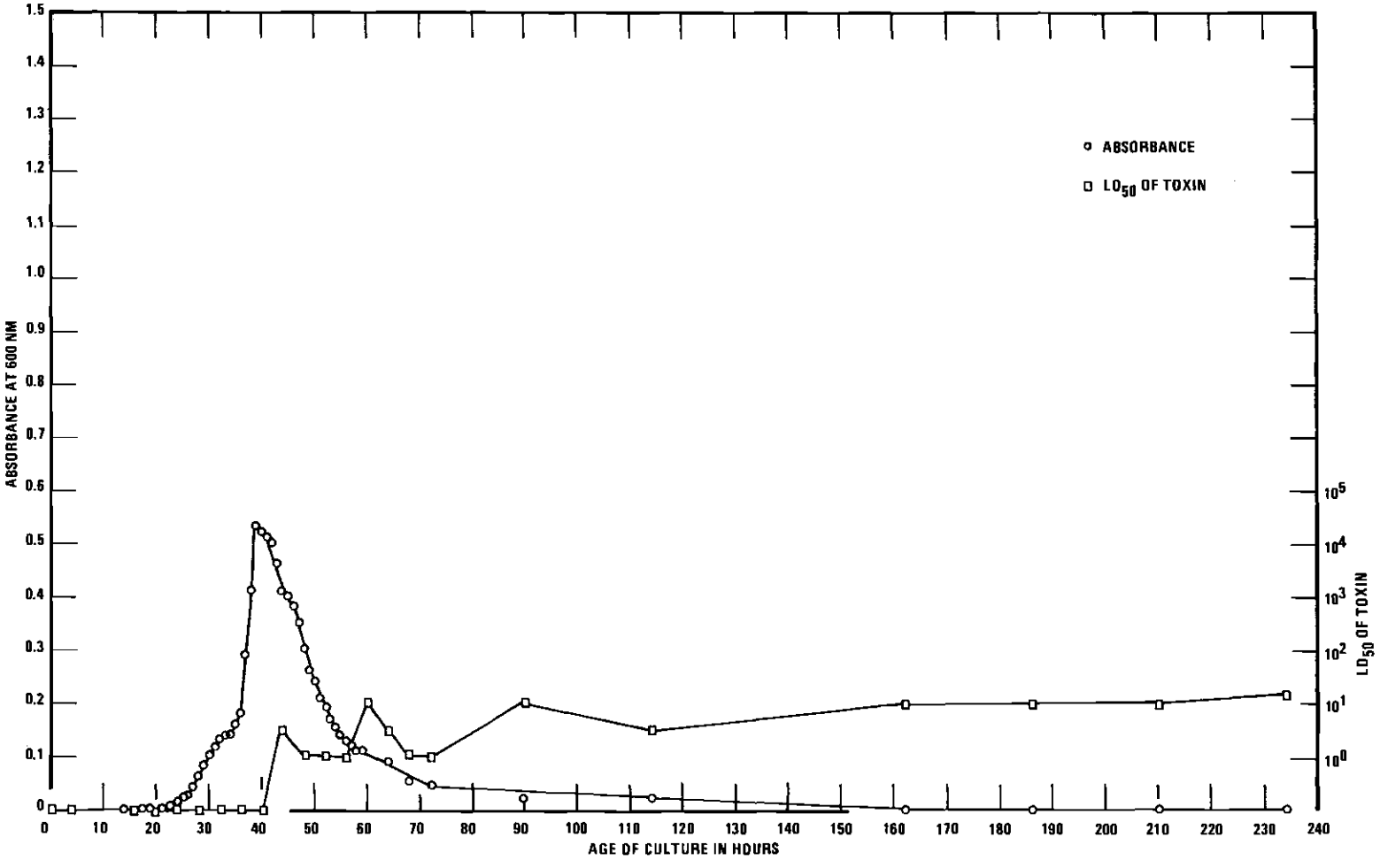

Figure 11. Effect of 0.1 Megarad of Cesium-137 Gamma Irradiation on Growth and Toxin Production of Spores of Clostridium botulinum Type F, Strain Craig in Trypticase Soy Broth at 25 C (Experiment 4).

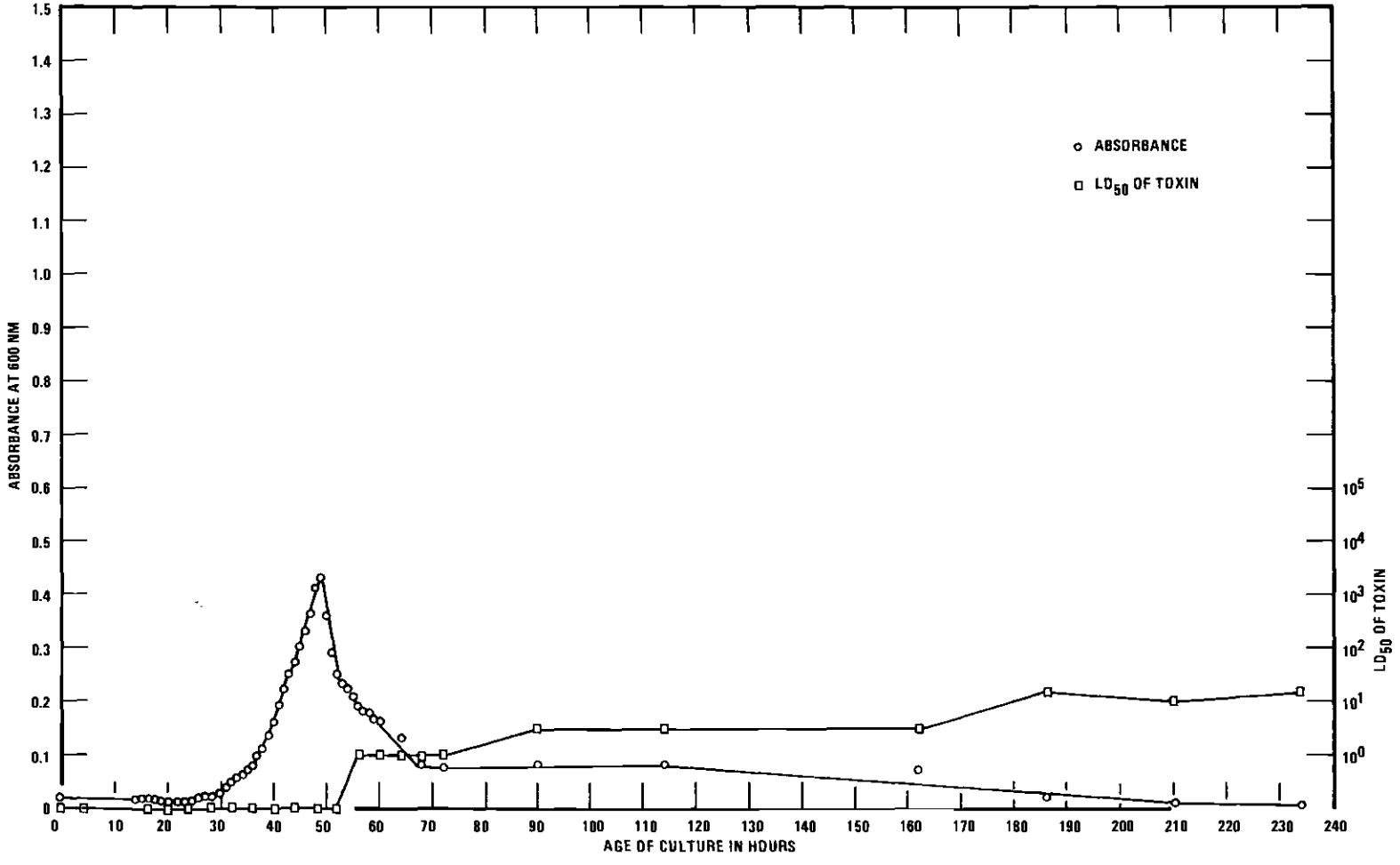

Figure 12. Effect of 0.2 Megarad of Cesium-137 Gamma Irradiation on Growth and Toxin Production of Spores of Clostridium botulinum Type F, Strain Craig in Trypticase Soy Broth at 25 C (Experiment 4).

broth at 25 C of irradiated cultures and an unirradiated control culture of the non-proteolytic Craig strain of Clostridium botulinum type F. The unirradiated, 0.1 megarad irradiated, and 0.2 megarad irradiated cultures of the Craig strain entered the logarithmic growth phase at 23, 22, and 24 hr, respectively, after inoculation into the growth medium (see Table 3). The unirradiated control culture continued in the logarithmic growth phase for 18 hr, reached a maximal optical density of 0.50, and began to autolyze after 41 hr of incubation. The 0.1 megarad irradiated culture remained in logarithmic growth phase for 17 hr, reaching a maximal optical density of 0.53 before undergoing autolysis after 39 hr. The 0.2 megarad irradiated culture reached a maximal optical density reading of 0.43 after 25 hr in the logarithmic growth phase and began to autolyze after 49 hr of incubation.

Toxin was first detected in the 0.1 megarad culture at 44 hr, in the 0.2 megarad culture at 56 hr, and not until 162 hr in the unirradiated control culture (see Table 4). Higher toxin titers were consistently detected in the irradiated cultures and these persisted over a 234 hr period. Trypsinization at pH 6.0 of the culture supernatant fractions did not result in earlier detection of toxin.

The results of a second experiment (experiment number 5) are shown in Figure 13, 14, and 15 and these data corroborate the data obtained previously. The cultures from unirradiated and irradiated spores remained in lag phase more than 22 hours. The cultures from unirradiated and 0.1 megarad irradiated spores entered the logarithmic growth phase at 23 hr after inoculation into the growth medium while the culture from 0.2 megarad irradiated spores entered the logarithmic

Table 3. Summary of Germination Times for 0.0, 0.1, and 0.2 Megarad Doses of Gamma Irradiation in Experiments on Clostridium botulinum Type F, Strain Craig Incubated at 25 C.

Experiment Number	Germination Time In Hours		
	0.0 Megarad	0.1 Megarad	0.2 Megarad
Four	23	22	24
Five	23	23	25

Table 4. Maximum Absorbance and LD_{50} Obtained in Experiments on *Clostridium botulinum* Type F, Strain Craig Incubated at 25 C.

Radiation Dosage	Experiment Number	Maximum OD Obtained	Age of Culture (Hrs.)	Initial Appearance of Toxin (Hrs.)	Maximum LD_{50} of Toxin	Age of Culture (Hrs.)
0.0 Megarad	4.	0.50	41	162	10.00	186
	5.	0.23	37	186	5.00	234
0.1 Megarad	4.	0.53	39	44	20.00	234
	5.	0.35	40	52	50.00	162
0.2 Megarad	4.	0.43	49	56	20.00	186
	5.	0.20	40	56	50.00	210

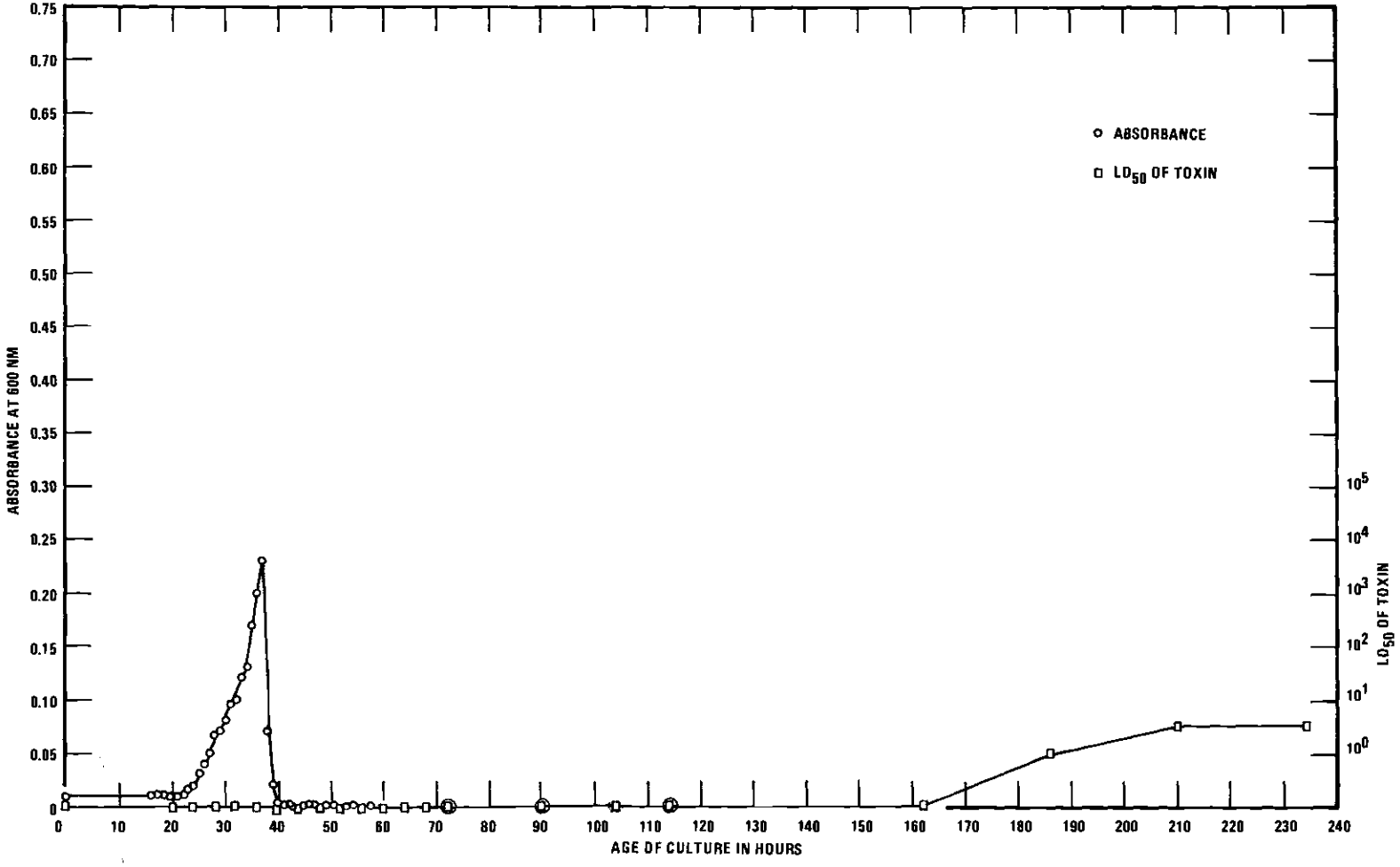

Figure 13. Growth and Toxin Production of Unirradiated Spores of Clostridim botulinum Type F, Strain Craig in Trypticase Soy Broth at 25 C (Experiment 5).

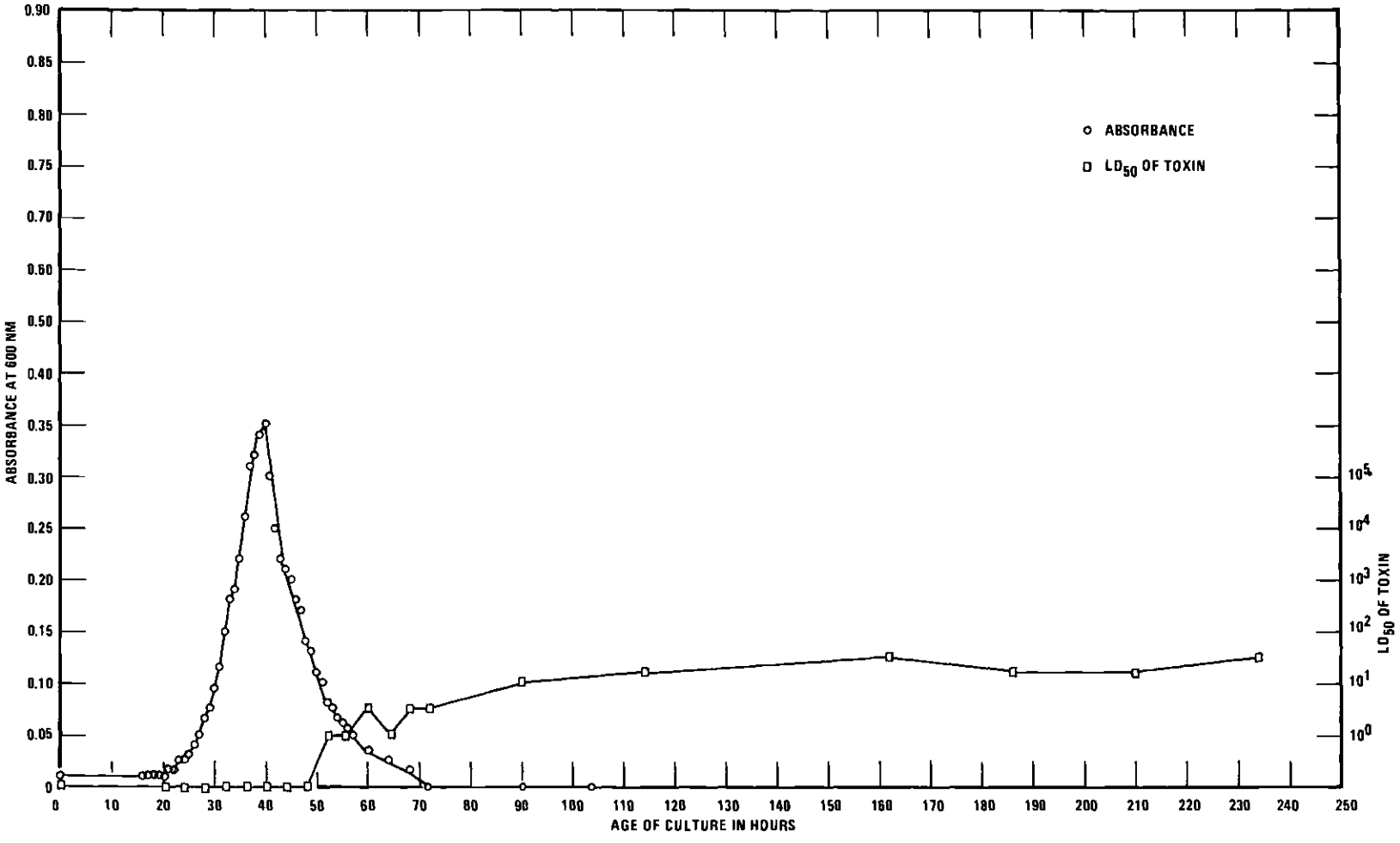

Figure 14. Effect of 0.1 Megarad of Cesium-137 Gamma Irradiation on Growth and Toxin Production of Spores of Clostridim botulinum Type F, Strain Craig in Trypticase Soy Broth at 25 C (Experiment 5).

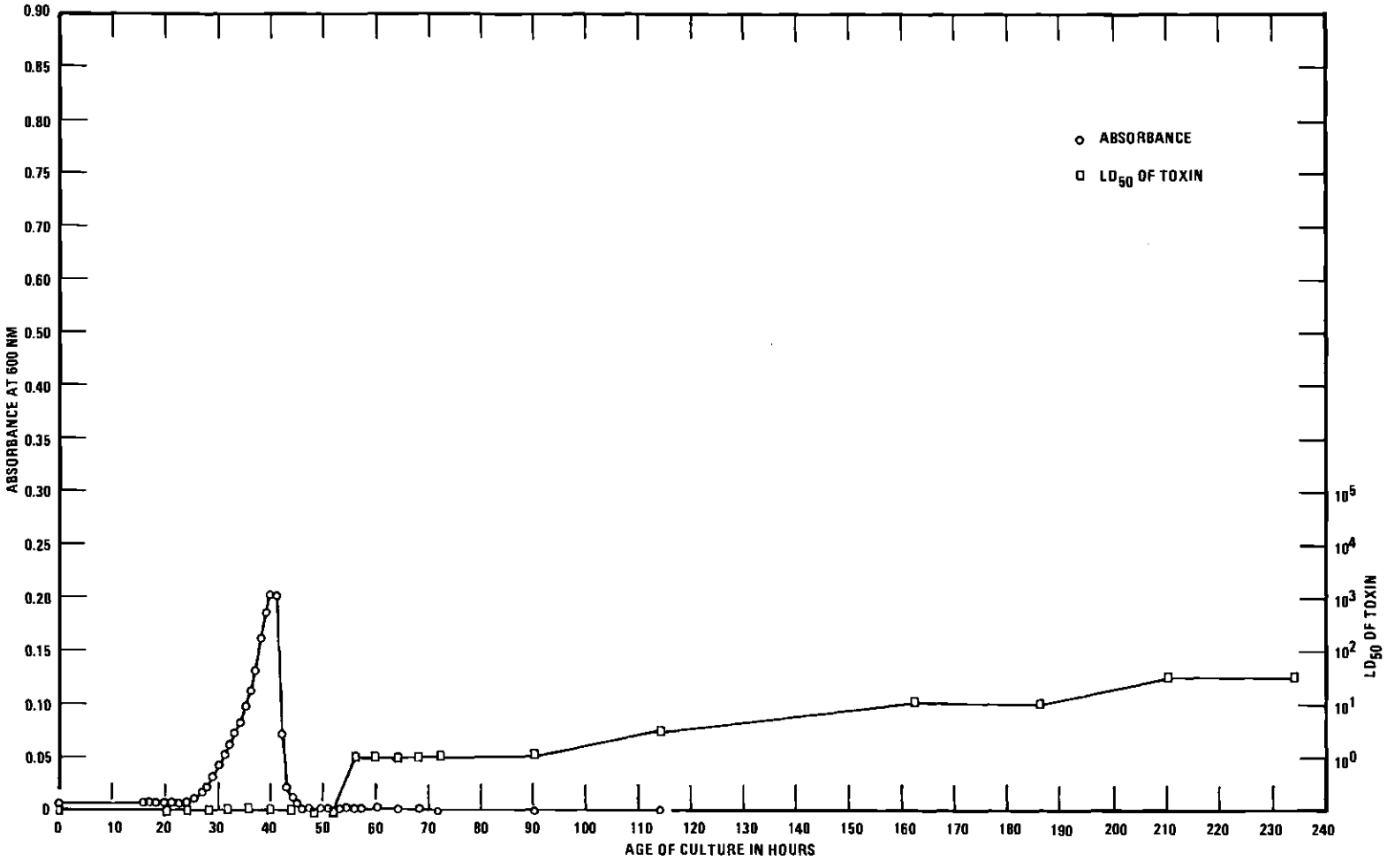

Figure 15. Effect of 0.2 Megarad of Cesium-137 Gamma Irradiation on Growth and Toxin Production of Spores of Clostridium botulinum Type F, Strain Craig in Trypticase Soy Broth at 25 C (Experiment 5).

growth phase after 25 hr (see Table 3). The unirradiated control culture continued in the logarithmic growth phase for the next 14 hr reaching a maximal optical density of 0.23 and then began to autolyze after 37 hr. The 0.1 megarad and 0.2 megarad irradiated cultures remained in the logarithmic growth phase 17 and 16 hr, respectively, reaching optical densities of 0.35 and 0.20 before undergoing autolysis after 40 and 41 hr, respectively.

Toxin was first detected at 52 hr in the 0.1 megarad irradiated culture and at 56 hr in the 0.2 megarad irradiated culture. The unirradiated control culture once again did not exhibit toxin until the 186 hr (see Table 4). In addition to the earlier appearance of the toxin in the irradiated cultures, toxin titers were also consistently higher in the irradiated cultures than in the unirradiated control throughout the experiment. Trypsin activation at pH 6.0 once again did not result in earlier detection of toxin.

B. Growth and Toxin Production at 10 C.

When the post-irradiation incubation temperature of cultures was 10 C, spore outgrowth of the Craig strain of Clostridium botulinum type F occurred only in the unirradiated and 0.1 megarad irradiated cultures (see Figure 16) although some spores did show signs of germination in the 0.2 megarad culture, as determined by microscopic examination. A maximal optical density of 0.09 was reached in the 0.1 megarad irradiated culture at 15 days while a maximal optical density of 0.07 was attained in the unirradiated control culture after 17 days incubation (see Table 5).

Undiluted culture supernatant from the 0.1 megarad irradiated

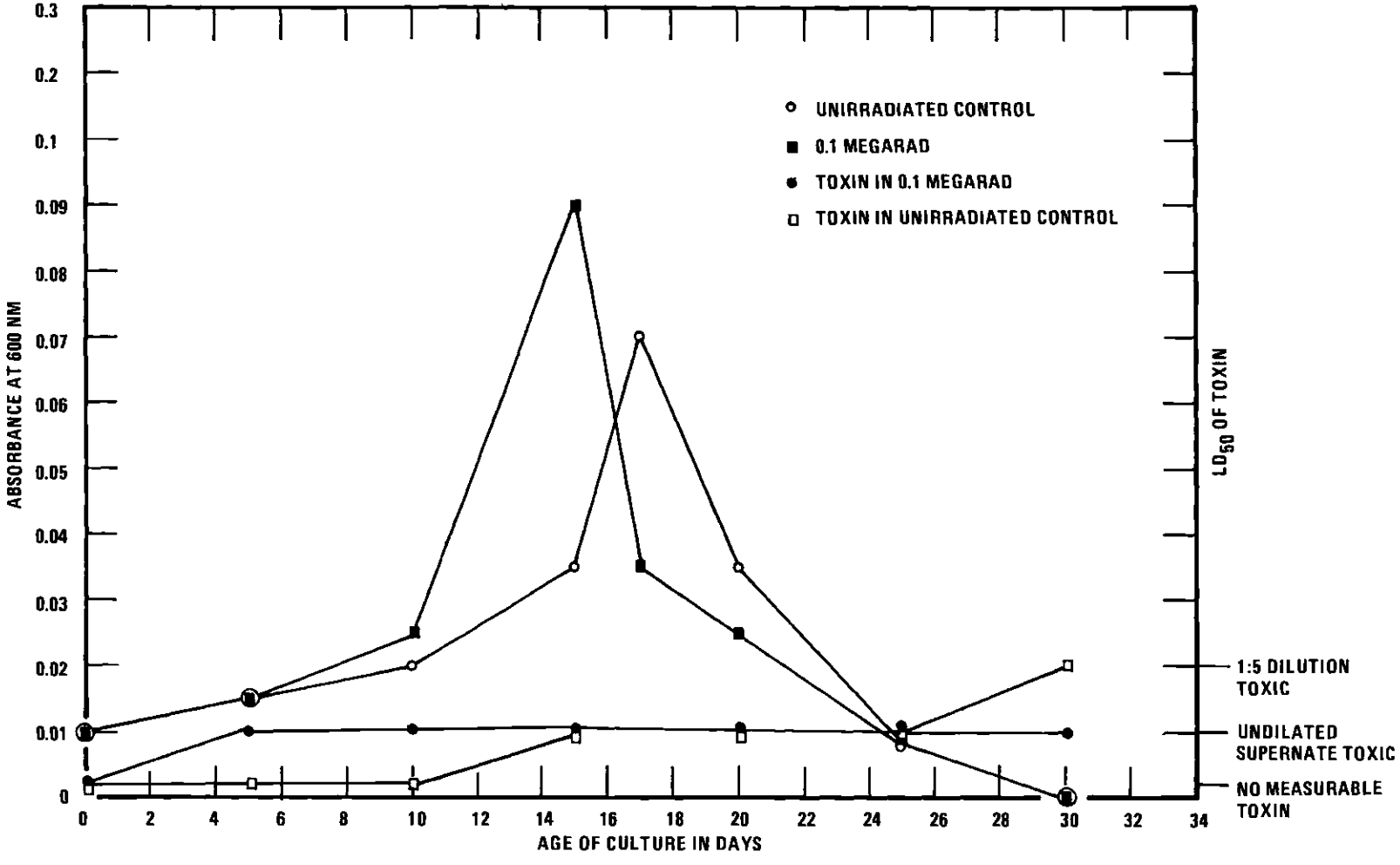

Figure 16. Growth and Toxin Production of Unirradiated and Irradiated Spores (0.1 Megarad of Cesium-137 Gamma Irradiation) of Clostridium botulinum Type F, Strain Craig in Trypticase Soy Broth at 10 C (Experiment 4).

Table 5. Maximum Absorbance and LD_{50} Obtained in Experiments on *Clostridium botulinum* Type F, Strain Craig Incubated at 10 C.

Radiation Dosage	Experiment Number	Maximum OD Obtained	Age of Culture (Days)	Initial Appearance of Toxin (Days)	Maximum LD_{50} of Toxin	Age of Culture (Days)
0.0 Megarad	4.	0.07	17	15	5.00	30
	5.	0.07	17	20	1.00	20
0.1 Megarad	4.	0.09	15	5	1.00	5
	5.	0.10	17	10	1.00	10

culture (experiment number 4) contained toxin after 5 days, whereas 15 days incubation were required before toxin could be detected in the unirradiated control culture. The low toxin level remained stable through 30 days in the 0.1 megarad irradiated culture. In the unirradiated control culture a slight increase in the toxin titer (LD_{50} = 5) was observed on the 30th day of incubation (see Table 5).

In the second experiment (experiment number 5) on the Craig strain at 10 C, spore outgrowth once again occurred only in the unirradiated and the 0.1 megarad irradiated cultures (see Figure 17) although some spores did show signs of germination in the 0.2 megarad irradiated culture, as determined by microscopic examination. The highest optical density reading (0.10) was reached at 17 days in the 0.1 megarad irradiated culture. The unirradiated control culture attained a peak optical density of 0.07 at 17 days (see Table 5).

Undiluted culture supernatant from the 0.1 megarad irradiated culture contained toxin after 10 days incubation at 10 C. Twenty days incubation were required before toxin was detected in the unirradiated control culture. This toxin level persisted through 30 days in both cultures (see Figure 17).

III. Survival of Spores of Clostridium botulinum Type F (I-8G-F and Craig) Subjected to 0.0, 0.1, or 0.2 Megarad Doses of Gamma Irradiation.

An experiment was performed to assay the number of spores capable of outgrowth and macroscopically visible colony formation immediately after irradiation. This was done to test the possibility that the increased cell mass of the irradiated cultures might be due to greater initial numbers of spores germinating because of an activation effect

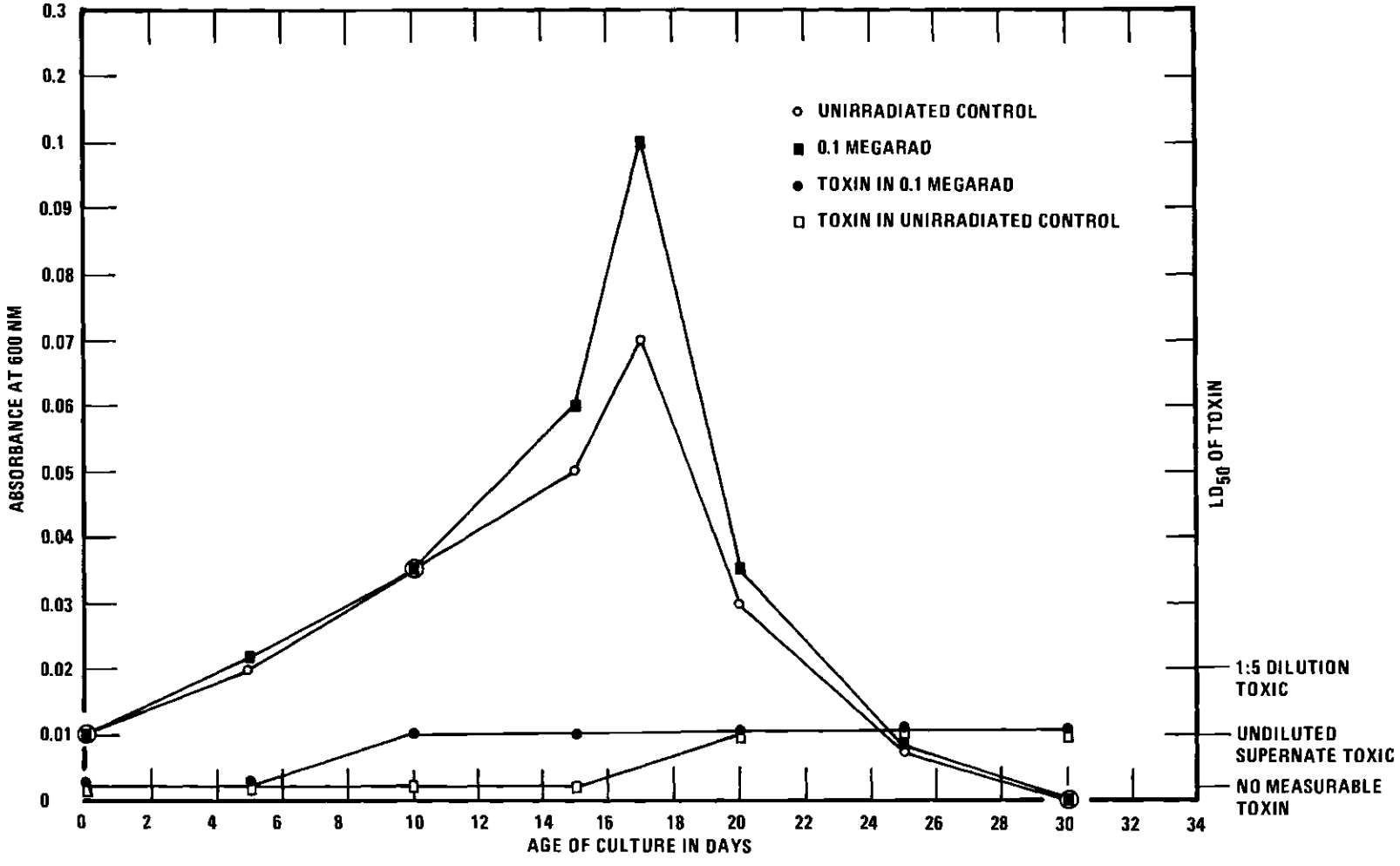

Figure 17. Growth and Toxin Production of Unirradiated and Irradiated Spores (0.1 Megarad of Cesium-137 Gamma Irradiation) of Clostridium botulinum Type F, Strain Craig in Trypticase Soy Broth at 10 C (Experiment 5).

of the gamma irradiation. Figure 18 shows the colony counts for the three irradiation experiments on the I-8G-F strain. At the levels of irradiation used, the number of spores capable of germination and subsequent outgrowth was unchanged.

Figure 19 shows the colony counts for the two irradiation experiments using the Craig strain of Clostridium botulinum type F. At the levels of radiation used, the 0.1 megarad dose caused an activation phenomenon while the 0.2 megarad dose resulted in an appreciable reduction in viable spores. This was reflected in the growth responses of these cultures. The 0.1 megarad irradiated cultures exhibited consistently higher optical density readings than the unirradiated control cultures while the 0.2 megarad irradiated cultures exhibited consistently lower optical density readings.

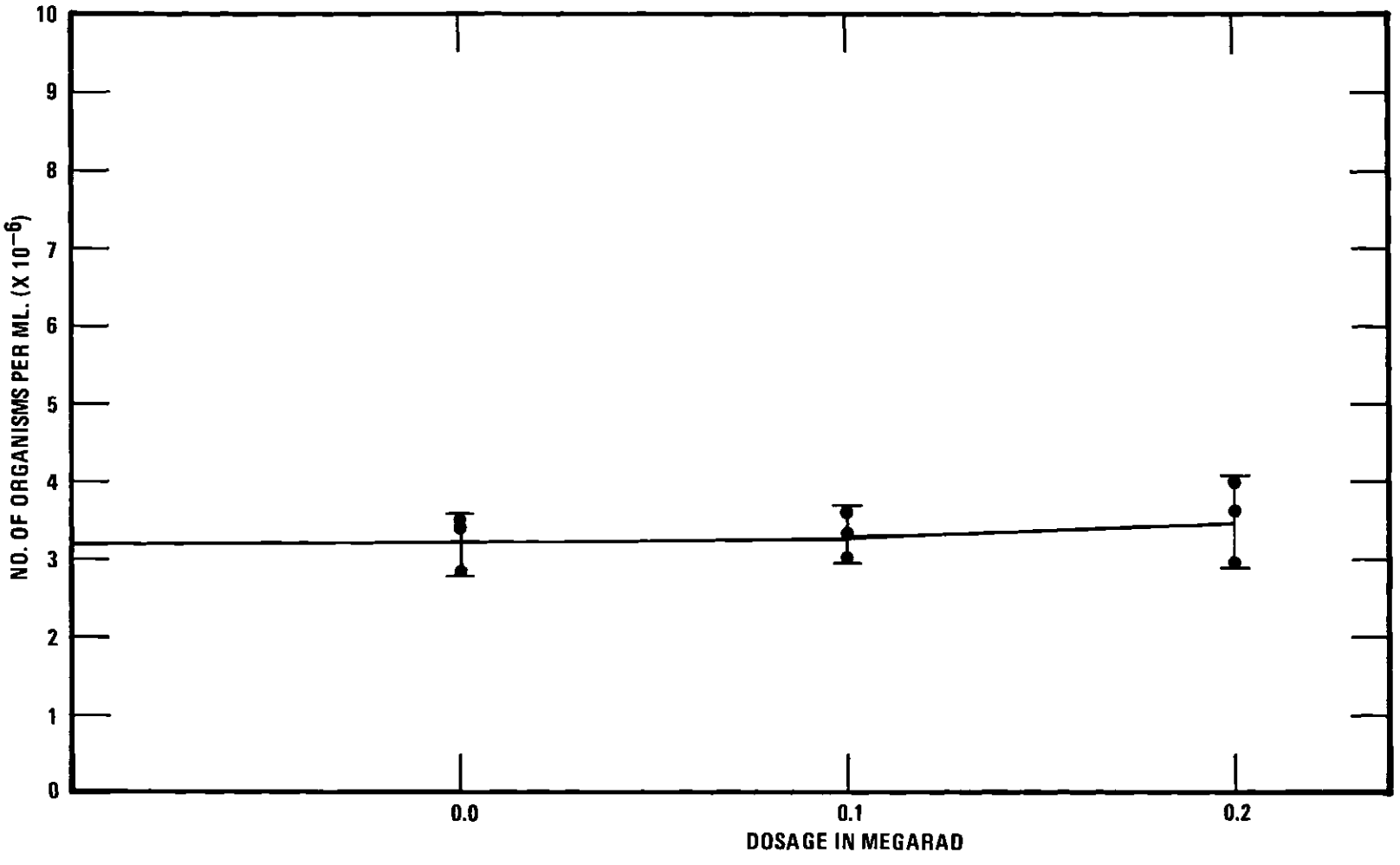

Figure 18. Effect of Cesium-137 Gamma Irradiation on Survival of Spores of Clostridium botulinum Type F, Strain I-8G-F Suspended in Sterile Sorenson's Phosphate Buffer pH 7.0 During Irradiation.

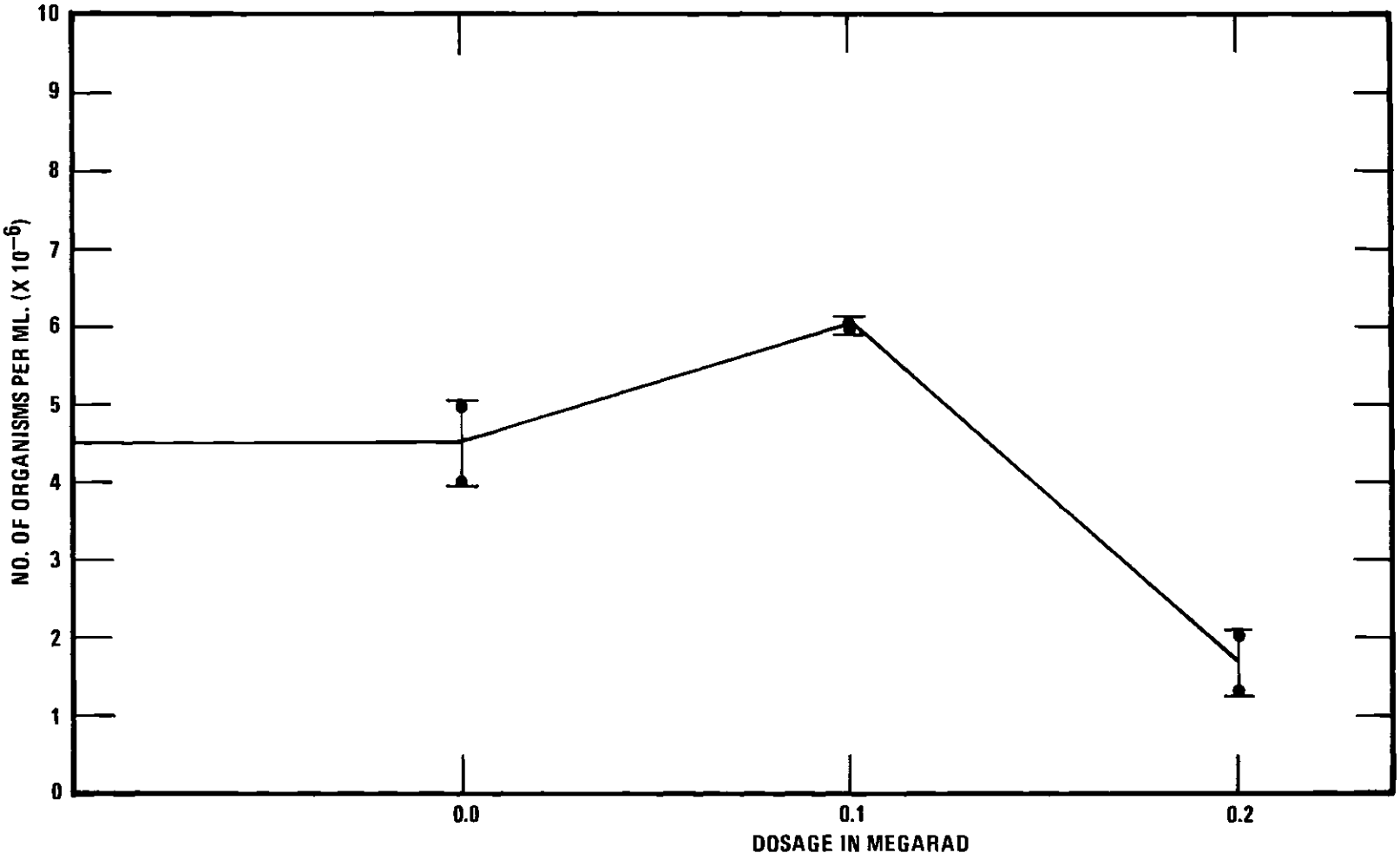

Figure 19. Effect of Cesium-137 Gamma Irradiation on Survival of Spores of Clostridium botulinum Type F, Strain Craig Suspended in Sterile Sorenson's Phosphate Buffer pH 7.0 During Irradiation.

CHAPTER IV

DISCUSSION

On the basis of the data obtained in the three experiments using the I-8G-F strain of Clostridium botulinum type F, the post-irradiation growth response of this organism at 30 C appears variable.

In the second experiment using this strain, all three cultures underwent partial lysis 9 to 12 hours after entering the logarithmic growth phase. Seven to fourteen hours were then required for the cultures to reach the same optical densities observed before the lytic phenomenon occurred. This same phenomenon had been observed with the Langeland strain of Clostridium botulinum type F (Williams-Walls, 1969). Dolman and Murakami (1961) noted a lytic effect while growing type F Clostridium botulinum on solid medium and they postulated that this was closely related to the dissociation or mutational tendencies of the organism. The presence of bacteriophage has also been suggested as a possible explanation for this lytic phenomenon (Williams-Walls, 1969). At present, the exact mechanisms involved are unknown and the present research offers no data from which more definite conclusions can be drawn. It does show that the phenomenon occurs in more than one strain of Clostridium botulinum type F growing in liquid media.

The experimental conditions present during the third experiment using the I-8G-F strain differed from those maintained during the first experiment in that the post-irradiation growth temperature was 35 C

instead of 30 C. This higher temperature resulted from a mechanical failure in the incubator. Under this altered condition, the total toxin produced by the irradiated cultures was not significantly greater than in the control culture. A possible reason for this may be that the increased post-irradiation growth temperature has a deleterious effect on a possible radiation damage-repair mechanism of the organism. Again, there is insufficient evidence either here or in the literature at the present time to support more than speculation on this point. Investigation of radiation damage-repair mechanisms was not the purpose of this research so this result was not pursued. It does offer potential for future studies, however.

On the basis of the data obtained in the two experiments using the Craig strain of Clostridium botulinum type F, the post-irradiation growth response of this organism at 25 C was enhanced by a radiation dose of 0.1 megarad and decreased by a radiation dose of 0.2 megarad. Cultures of the Craig strain from spores subjected to 0.1 megarad of gamma irradiation exhibited higher optical densities than the unirradiated controls while cultures from spores subjected to 0.2 megarad of gamma irradiation exhibited lower optical densities than the unirradiated controls. The higher optical densities of cultures from 0.1 megarad irradiated spores may have resulted from an activation phenomenon. These responses might be the result of a disturbance in the protein metabolism of cultures from irradiated spores. The lower optical densities observed in cultures from 0.2 megarad irradiated spores at 25 C may be explained by the lower radioresistance of this strain. Previously established radiation survival curves have shown that this organism is less radioresistant than

some of the proteolytic strains of Clostridium botulinum.

The post-irradiation growth temperatures of 25 C for the Craig strain and 30 C for the I-8G-F strain of Clostridium botulinum type F were selected because these temperatures produce optimal growth of the organisms in terms of cell mass.

To assure that the increased optical density readings were not the result of clumping or increased cell size, microscopic examinations of the cultures were made hourly after spore inoculation into culture media. No apparent difference in individual cell size and no evidence of cell clumping was observed throughout the experimentation.

The data in the experiments performed at 25 C and 30 C were subjected to a t test (Goldstein, 1964) to ascertain the significance of a difference between two slopes. Statistical treatment of these data verified no significant differences in the slopes of the regression lines of the unirradiated and irradiated cultures in each individual experiment and also between different experiments at the 99 percent confidence level (see Appendix B). The statistical treatment of the data confirms the conclusion that there is no difference in the rate of growth of the unirradiated and irradiated cultures.

At an incubation temperature of 10 C, spores of the proteolytic I-8G-F strain failed to multiply after irradiation. The proteolytic I-8G-F strain of Clostridium botulinum type F will not grow at temperatures below 15 C. This organism is a mesophilic organism having a growth range between 10 C-45 C, maximum growth occurring at 35 C. Thus, the negative results obtained at 10 C were not totally unexpected.

At an incubation temperature of 10 C, spores of the Craig strain

treated with 0.2 megarad gamma irradiation failed to multiply after germination. This was probably due to subjection of the culture to a second unfavorable treatment (sub-optimal temperature) after irradiation. Spores subjected to 0.1 megarad gamma irradiation prior to incubation at 10 C, however, showed more growth and earlier production of toxin than detected in the unirradiated control culture.

In the studies on toxin production by both proteolytic and non-proteolytic strains of Clostridium botulinum type F after exposure to varying levels of ionizing radiation, toxin was measured every 4 hours from initiation of the experiments (0 time) to 72 hr and every 24 hr thereafter to 164 hr for cultures of the I-8G-F strain incubated at 30 C, and to 234 hr for cultures of the Craig strain incubated at 25 C. Toxin was measured every 5 days for a 30 day period for all cultures incubated at 10 C. No toxin was demonstrable in unirradiated and irradiated spore suspensions, in culture supernatant fractions prior to germination, or in uninoculated culture media and trypsinization of these samples did not produce detectable toxin.

Irradiation of spores of Clostridium botulinum type F, strain I-8G-F did not cause earlier appearance of toxin in cultures grown from these spores but it did affect the LD_{50} toxin titers of the cultures. Radiation treatment may cause a disturbance in the protein metabolism of the irradiated cells which results in increased toxin production during post-irradiation growth. This could be due to either an alteration of the amount of toxin produced or an increased toxicity of the toxin produced by cells in these cultures. These higher toxin titers persisted over extended periods of experimentation, indicating the

stability of this increased titer.

In the non-proteolytic Craig strain of Clostridium botulinum type F, toxin is not detectable until 6 to 7 days after inoculation of spores or cells into a culture medium incubated at 25 C (N. W. Walls, unpublished data). The reason for this is not understood. However, it has been postulated that this amount of time is required for the toxin to become activated due to either some enzymatic interaction or to uncoiling of the molecule with age. As with the I-8G-F strain of Clostridium botulinum type F, radiation treatment at both the 0.1 megarad and 0.2 megarad levels with cesium 137 gamma rays altered this response. It was observed in the two experiments on the Craig strain of Clostridium botulinum type F that toxin was first detected at 44 hr and 52 hr in the 0.1 megarad irradiated cultures, at 56 hr in both 0.2 megarad irradiated cultures, and not until 162 hr and 186 hr in the unirradiated controls.

The data on survival of spores of Clostridium botulinum type F, strain I-8G-F subjected to 0.0, 0.1, or 0.2 megarad doses of gamma irradiation showed that, at the levels of irradiation used, the number of spores capable of germination and subsequent outgrowth was unchanged. Thus division by greater initial numbers of viable spores is probably not the explanation of the observed growth response and toxin production from irradiated spores.

The data on survival of spores of Clostridium botulinum type F, strain Craig subjected to 0.0, 0.1, or 0.2 megarad doses of gamma irradiation indicated that the 0.1 megarad dose caused an activation phenomenon. The activation phenomenon was accompanied by consistently

higher optical density readings and earlier toxin production in cultures grown at 25 C from the 0.1 megarad irradiated spores compared to control cultures. These responses also may be the result of a disturbance in the protein metabolism of cultures from irradiated spores.

Exposure of spores of the Craig strain to a 0.2 megarad dose of ionizing radiation caused an appreciable reduction in viable spores. These colony counts and, subsequently, the growth response of the 0.2 megarad irradiatiated spores at 25 C may be explained by the lower radio-resistance of this strain. Previously established radiation survival curves for this strain have shown that this organism does not demonstrate the characteristic "shoulder" as observed with proteolytic strains of Clostridium botulinum. Therefore a reduction in number of viable spores at a radiation level of 0.2 megarad was expected.

In conclusion, this research shows that radiation treatment of spores of Clostridium botulinum type F results in a post-irradiation growth response for the I-8G-F and Craig strains at optimum growth temperature that is strain dependent. Consistently higher toxin titers are produced at optimum growth temperatures by cultures from irradiated spores than from unirradiated controls in both strains, with the Craig strain also manifesting an earlier production of toxin in irradiated cultures. At a sub-optimum incubation temperature of 10 C, spores of the proteolytic strain of Clostridium botulinum failed to grow and in the non-proteolytic strain only the cultures previously subjected to 0.0 or 0.1 megarad gamma irradiation grew. At this temperature the 0.1 megarad irradiated cultures demonstrated an increased growth response and earlier toxin production than the unirradiated control.

An interrelationship between temperature and irradiation versus growth and toxin production is suggested by the data collected. If the fact that optical density readings are logarithmic is considered, it appears that at 10 C there is more toxin produced per cell mass of Clostridium botulinum type F, strain Craig than at 25 C. It might be that the optimum temperature for production of cell mass; i.e., 25 C, preferentially favors the complex metabolic processes involved in production of cell material by the organism while at a temperature of 10 C production of cellular components by the organism is impeded and toxin synthesis therefore appears to have increased with decreasing temperature. This would also be a fruitful area for more quantitative studies.

CHAPTER V

CONCLUSIONS

Based on the research reported in the preceding text, the following conclusions are made:

The post-irradiation growth response at 30 C in TSB of spores of Clostridium botulinum type F, strain I-8G-F subjected to 0.1 megarad and 0.2 megarad cesium-137 gamma irradiation as compared to an unirradiated control culture is variable.

The toxin titers produced at 30 C by cultures from irradiated spores of Clostridium botulinum type F, strain I-8G-F are consistently higher than those produced by an unirradiated control culture.

No spore outgrowth or toxin production occurs at 10 C in the 0.0, 0.1, or 0.2 megarad irradiated cultures of Clostridium botulinum type F, strain I-8G-F.

The post-irradiation growth response at 25 C in TSB of spores of Clostridium botulinum type F, strain Craig is greater in the 0.1 megarad irradiated culture and lower in the 0.2 megarad irradiated culture than in the unirradiated control culture.

Gamma irradiation of spores of Clostridium botulinum type F, strain Craig with 0.1 megarad or 0.2 megarad results in earlier toxin production and consistently higher toxin titers than produced by the unirradiated control culture at 25 C.

The post-irradiation growth response at 10 C in TSB of spores of Clostridium botulinum type F, strain Craig subjected to 0.1 megarad

gamma irradiation results in higher optical density readings than manifested by the unirradiated control culture. At 10 C toxin is produced earlier in the 0.1 megarad irradiated culture than in the unirradiated control culture.

At 10 C no spore outgrowth occurs in the 0.2 megarad irradiated culture of Clostridium botulinum type F, strain Craig.

CHAPTER VI

RECOMMENDATIONS

Based on the results of this investigation, the following recommendations are submitted for further consideration:

1. A more comprehensive determination of growth response and toxin production at 10 C. This type of experimentation would be of considerable importance to the food industry.

2. A detailed study of the factors which induce the partial lysis phenomenon of growing cells of Clostridium botulinum. Of particular interest is the effect of this partial lysis phenomenon on toxin titers.

3. A more accurate correlation of cell mass and numbers in relation to specific optical density readings. Optical density is an indirect measure of cell mass and does not indicate the absolute number of cells present. A more accurate measure of cell mass can be obtained by centrifuging the cells in calibrated cuvettes and determining the cell volume. These cell volumes could then be compared to specific optical density readings. This information could be compared with actual counts of cell numbers present using a Coulter counter.

4. Studies employing the Technicon Auto-Analyzer to determine optimum growth conditions and to produce more accurate growth curves and toxin production data. Manual manipulations of the Nephelo flasks used in the present study cause disturbance of the growth medium and reduction of the anaerobic environment.

APPENDICES

APPENDIX A

Cooked Meat Medium

The commercial preparation of Cooked Meat Medium (Difco) was used. Depending upon the amount of medium desired, a quantity of the dehydrated cooked meat medium was suspended in cold deionized water, in screw cap tubes or flasks. The medium was mixed thoroughly and allowed to stand 15 minutes so that all the particles were thoroughly wetted, and maintaining an even suspension. The medium was sterilized in the autoclave for 15 minutes at 15 psi (121 C), then allowed to cool to room temperature without agitation. The pH of the medium after sterilization was 7.2.

Pork Infusion Medium

Fresh pork infusion medium is prepared in the following manner. A fresh lean pork ham weighing between 10-12 pounds is used. A 12 pound ham produces approximately 8 liters of medium.

First Day

1. Remove the meat from the bone, stripping off the fat. Then put the meat through a grinder, using the fine attachment.
2. For each pound of ground ham, add one liter of cold tap water. Mix well, bring to a boil, then simmer for one hour, covered.
3. Remove from heat, allow to cool just enough to handle, then strain off the broth through eight layers of cheesecloth into a large clean container.
4. Allow the broth to cool to room temperature. Cover the

container and then refrigerate overnight.

Second Day

5. Skim the solidified fat from the top of the broth. Measure the broth and bring up to the original volume again with tap water if necessary.

6. Add to the broth:

   Bacto-Peptone..... 5g/liter

   Glucose..... 1g/liter

   Bacto-Tryptone.....1.5g/liter

   K_2HPO_4.....1.25g/liter

   Soluble Starch..... 1g/liter

   Sodium Thioglycollate (Difco)..... 1g/liter

   Heat the medium to dissolve the solids. Cool to room temperature and adjust to pH 7.4 with 10 N NaOH.

7. Divide the medium into convenient amounts in 3 or 4 liter Erlenmeyer flasks. Add 1.5 percent Bacto-Agar to each flask, plug with cotton and cover plug with aluminum foil. Autoclave for 30 minutes at 15 psi.

8. Remove the flasks to a water bath set at 55 C. Adjust to a tilted position to allow the precipitate which will form to collect in a small area at the bottom of the flask. Allow to remain overnight.

Third Day

9. Using care not to disturb the precipitate, decant the clear liquid in each flask through four layers of cheesecloth, with one-half inch of cotton between two of the layers. Keep the

receiving vessel in hot water to prevent hardening. Dispense into modified agar slant tubes (about 25 ml per tube), cotton plug and place in tall round baskets. Weight the top with a wodden disc cut slightly smaller than the basket opening and a brick, (to prevent blowing off the cotton plugs). Autoclave for 30 minutes at 15 psi, allow the agar to gel, and store in a cool place.

Gelatin Diluent (Duff, et al., 1957)

Bacto-Gelatin (Difco)..... 2g

$Na_2HPO_4 \cdot 7\ H_2O$..... 4g

Deionized water..... 1000ml

The ingredients were dissolved in deionized water, and the mixture then adjusted to a pH of 6.8 with dilute HCL (10%). The buffer was dispensed in 100 ml quantities into new screw cap, pint prescription bottles and autoclaved at 121 C (15 psi) for 15 minutes. The gelatin diluent was stored at room temperature until needed to dilute toxin samples.

Peptone Water

Bacto-Peptone..... 1g

Deionized water.....1000ml

The peptone was dissolved in water and 99 ml aliquots dispensed into 150 ml milk dilution bottles. After autoclaving for 20 minutes at 121 C and 15 psi, the bottles were stoppered with sterile rubber stoppers and stored at room temperature.

Schmidt's Counting Medium

Bacto-Peptone (Difco)..... 50g

Yeast Extract (BBL)..... 10g

Sodium Thioglycollate (Difco)..... 1g

Bacto-Agar (Difco).....10g

Deionized water..... 1000ml

All the dry ingredients except agar were added to the deionized water and the mixture heated with constant stirring until dissolved. After the liquid had cooled to room temperature the pH was adjusted to 7.0-7.2. The agar was then added to the medium, heated until it had dissolved, and then dispensed into modified agar slant tubes, 25 ml per tube. The tubes were cotton plugged and placed into tall round baskets. The tops of the tubes were weighted as described in the preparation of pork infusion agar. The medium was then autoclaved 15 minutes at 15 psi prior to use. Schmidt's medium was freshly prepared on the day it was used in an experiment.

Sorenson's Phosphate Buffer

M/15 Na_2HPO_4.....61.1ml

M/15 KH_2PO_4.....38.9ml

The phosphate buffer was dispensed into new screw cap pint medicine bottles. The combination of the two chemicals in these quantities should yield a pH of 7.0, which was confirmed with a Beckman pH meter. The buffer was then autoclaved at 121 C (15 psi) for 15 minutes and allowed to cool to room temperature. The phosphate buffer was stored at 4 C until used.

Spore Stain

An adaptation of Conklin's Modification of Wirtz's Method was employed to stain spores with malachite green dye:

1. Smears were prepared by standard techniques and heat fixed.
2. The slides were flooded with 5 percent aqueous malachite green dye, and steamed for 5 minutes. Fresh stain was added to keep the dye from drying on the slides.
3. Slides were washed with tap water until no more dye came off.
4. Slides were counterstained for 1 minute with 1 percent safranin.
5. Slides were then washed in tap water and allowed to air dry.

Under microscopic examination, the highly refractive spores appear bright green in color while the vegetative cells appear red.

As spores begin to germinate, they start to lose their refractivity and begin to take up methylene blue dye. This stage of development can be detected by staining smeared preparations with methylene blue stain.

1. Smears were prepared by standard techniques and heat fixed.
2. The slides were flooded for two minutes with methylene blue.
3. Slides were washed for 30 seconds in running tap water and allowed to air dry.

Spores undergoing germination are deep blue in color.

Trypticase Soy Agar (BBL)

Sixty grams of the dehydrated trypticase soy agar were suspended in 1500 ml of deionized water in a 3200 ml modified Fernbach flask. The medium was heated with frequent agitation and boiled for one minute. The flask was then stoppered with a cotton plug wrapped in one layer of cheesecloth, overlaid with aluminum foil, and autoclaved at 121 C (15 psi) for 15 minutes. The agar was then allowed to solidify in the flask at room temperature.

Trypticase Soy Broth (BBL)

Fifteen grams of the dehydrated powder were suspended in 500 ml deionized water in a one liter Erlenmeyer flask. The medium was mixed thoroughly and then warmed gently until solution was complete. The pH of the medium was adjusted to pH 7.3 and then the flask was plugged with a cotton plug wrapped in one layer of cheesecloth and overlaid with aluminum foil. The medium was autoclaved at 121 C (15 psi) for 15 minutes, allowed to cool at room temperature. It was gently poured onto the hardened TSA base in the Fernbach flask. When used as a growth medium the TSB was prepared as above, poured into Nephelo culture flasks in 244 ml aliquots, and autoclaved in these flasks.

Type C Toxin Medium (Cardella et al., 1958)

Proteose Peptone (Difco)..... 40g

N-Z-Amine Type B (Sheffield Farms)..... 20g

Yeast Extract (BBL)..... 20g

Dextrose..... 10g

Deionized water.....950ml

The ingredients (except dextrose) were added to the deionized water and the mixture heated with constant stirring until they were dissolved. After the liquid had cooled to room temperature, the pH was adjusted to 7.2 with 10 N NaOH and the medium dispensed into Erlenmeyer flasks. These were plugged with cotton wrapped in one layer of cheesecloth and covered with aluminum foil. The medium was autoclaved at 121 C and 15 psi for 30 minutes and then rapidly cooled in an ice bath until warm to the touch. Filter-sterilized dextrose was added aseptically just prior to inoculation, to give a final concentration of 1 percent, and the flasks gently swirled in order to distribute the dextrose throughout the medium.

APPENDIX B

Statistical Analysis

Symbols Used in Statistical Analysis

x: hours of incubation following inoculation.

y: absorbance obtained at the corresponding value for hours of incubation.

N: the number of items in a sample.

Σ: upper-case Greek sigma, meaning "take the sum of".

b: slope of the regression line.

SS_x: sum of the squares of the x values.

$S_{y \cdot x}$: a standard deviation of individual y values about the regression line.

Table 6. Computation of Values Required for Analysis of Significance of Difference Between Two Slopes of the First Experiment on Unirradiated Spores of Clostridium botulinum Type F, Strain I-8G-F.

Case	x	x^2	y	y^2	xy
1	18	324	0.08	0.0064	1.44
2	19	361	0.15	0.0225	2.85
3	20	400	0.23	0.0529	4.60
4	21	441	0.35	0.1225	7.35
5	22	484	0.42	0.1764	9.24
6	23	529	0.58	0.3364	13.34
7	24	576	0.75	0.5625	18.00
8	25	625	0.85	0.7225	21.25
9	26	676	0.95	0.9025	24.70
10	27	729	1.00	1.0000	27.00
11	28	784	1.046	1.0941	29.29
12	29	841	1.097	1.2034	31.81
Total	282	6770	7.503	6.2021	190.87

Table 7. Computation of Values Required for Analysis of Significance of Difference Between Two Slopes of the First Experiment on Spores of Clostridium botulinum Type F, Strain I-8G-F Irradiated with 0.1 Megarad Gamma Irradiation.

Case	x	x^2	y	y^2	xy
1	16	256	0.02	0.0004	0.32
2	17	289	0.04	0.0012	0.60
3	18	324	0.07	0.0049	1.26
4	19	361	0.12	0.0149	2.32
5	20	400	0.20	0.0400	4.00
6	21	441	0.28	0.0784	5.88
7	22	484	0.45	0.2025	9.90
8	23	529	0.64	0.4096	14.72
9	24	576	0.70	0.4900	16.80
10	25	625	0.85	0.7225	21.25
11	26	676	0.95	0.9025	24.70
12	27	729	1.00	1.0000	27.00
13	28	784	1.046	1.0941	29.29
14	29	841	1.097	1.2034	31.81
Total	315	7315	7.46	6.1644	189.85

Table 8. Computation of Values Required for Analysis of Significance of Difference Between Two Slopes of the First Experiment on Spores of Clostridium botulinum Type F, Strain I-8G-F Irradiated with 0.2 Megarad Gamma Irradiation.

Case	x	x^2	y	y^2	xy
1	16	256	0.01	0.0001	0.16
2	17	289	0.02	0.0004	0.34
3	18	324	0.03	0.0009	0.54
4	19	361	0.05	0.0025	0.95
5	20	400	0.10	0.0100	2.00
6	21	441	0.15	0.0225	3.15
7	22	484	0.26	0.0676	5.72
8	23	529	0.38	0.1444	8.74
9	24	576	0.45	0.2025	10.80
10	25	625	0.62	0.3844	15.50
11	26	676	0.75	0.5625	19.50
12	27	729	0.85	0.7225	22.95
13	28	784	0.95	0.9025	26.60
14	29	841	1.00	1.0000	29.00
Total	315	7315	5.62	4.02	145.95

Computation of significance of difference between slopes obtained during the experiments on Clostridium botulinum type F, strains Craig and I-8G-F was determined in the following manner.

Computation of Significance of Difference Between Two Slopes of the First Experiment on Clostridium botulinum Type F, Strain I-8G-F.

Unirradiated Spores

$$\text{Slope} = \frac{\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{N_2}}{\Sigma x^2 - \frac{(\Sigma x)}{N}}$$

$$= \frac{190.87 - \frac{(282)(7.503)}{12}}{6770 - \frac{(282)^2}{12}} = 0.102$$

$$\text{Sum of the Squares} = \Sigma x^2 - \frac{(\Sigma x)^2}{N}$$

$$= 6770 - \frac{(282)^2}{12} = 143$$

Standard Deviation of Individual y-values About the Regression

$$\text{Line} = \sqrt{\frac{1}{N-2}\left(\Sigma y^2 - \frac{(\Sigma y)^2}{N} - b\left[\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{N}\right]\right)}$$

$$= \sqrt{\frac{1}{10}\left(6.2021 - \frac{(7.503)^2}{12} - 0.102\left[190.87 - \frac{(282)(7.502)}{12}\right]\right)}$$

$$= 0.055$$

Spores Irradiated With 0.1 Megarad

$$\text{Slope} = \frac{\Sigma xy - \frac{(\Sigma x)\ (\Sigma y)}{N}}{\Sigma x^2 - \frac{(\Sigma x)^2}{N}}$$

$$= \frac{189.85 - \frac{(315)\ (7.46)}{14}}{7315 - \frac{(315)^2}{14}} = 0.0967$$

$$\text{Sum of the Squares} = \Sigma x^2 - \frac{(\Sigma x)^2}{N}$$

$$= 7315 - \frac{(315)^2}{14} = 227.5$$

Standard Deviation of Individual y-values About the Regression

$$\text{Line} = \sqrt{\frac{1}{N-2}\left(\Sigma y^2 - \frac{(\Sigma y)^2}{N} - b\left[\Sigma xy - \frac{(\Sigma x)\ (\Sigma y)}{N}\right]\right)}$$

$$= \sqrt{\frac{1}{12}\left(6.1644 - \frac{(7.46)^2}{14} - 0.0967\left[189.85 - \frac{(315)\ (7.46)}{14}\right]\right)}$$

$$= 0.0693$$

Spores Irradiated with 0.2 Megarad

$$\text{Slope} = \frac{\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{N}}{\Sigma x^2 - \frac{(\Sigma x)^2}{N}}$$

$$= \frac{145.95 - \frac{(315)\ (5.62)}{14}}{7315 - \frac{(315)^2}{14}}$$

$$= 0.086$$

$$\text{Sum of the Squares} = \Sigma x^2 - \frac{(\Sigma x)^2}{N}$$

$$7315 - \frac{(315)^2}{14} = 227.5$$

Standard Deviation of Individual y-values About the Regression

$$\text{Line} = \sqrt{\frac{1}{N-2}\left(\Sigma y^2 - \frac{(\Sigma y)^2}{N} - b\left[\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{N}\right]\right)}$$

$$= \sqrt{\frac{1}{12}\left(4.02 - \frac{(5.62)^2}{14} - 0.086\left[145.95 - \frac{(315)(5.62)}{14}\right]\right)}$$

$$= 0.085$$

Significance of Difference Between Slopes for Unirradiated Spores of Clostridium botulinum Type F, Strain I-8G-F Versus Spores Subjected to 0.1 Megarad in the First Experiment.

$$\text{Pooled Error Variance} = \sqrt{(S_{y\cdot x})_1^2 + (S_{y\cdot x})_2^2}$$

$$= \sqrt{(0.055)^2 + (0.0693)^2}$$

$$= 0.0883$$

$$t \text{ test} = \frac{b_1 - b_2}{S_{y\cdot x}\sqrt{\frac{1}{SS_{x_1}} + \frac{1}{SS_{x_2}}}}$$

$$= \frac{0.102 - 0.0967}{0.0883\sqrt{\frac{1}{143} + \frac{1}{227.5}}} = 0.5698$$

The t test value of 0.5698 is < 2.82 $n_1 = 12$, $n_2 = 14$

Therefore the slopes are not significantly different.

Significance of Difference Between Slopes for Unirradiated Spores of Clostridium botulinum Type F, Strain I-8G-F Versus Spores Subjected to 0.2 Megarad in the First Experiment.

$$\text{Pooled Error Variance} = \sqrt{(S_{y\cdot x})_1^2 + (S_{y\cdot x})_2^2}$$

$$= \sqrt{(0.055)^2 + (0.085)^2} = 0.1012$$

$$t \text{ test} = \frac{b_1 - b_2}{S_{y \cdot x} \sqrt{\frac{1}{SS_{x_1}} + \frac{1}{SS_{x_2}}}}$$

$$= \frac{0.102 - 0.0901}{0.1012 \sqrt{\frac{1}{143} + \frac{1}{227.5}}} = 1.1121$$

The t test value of 1.1121 is < 2.82 $n_1 = 12$, $n_2 = 14$

Therefore the slopes are not significantly different.

Significance of Difference Between Slopes for Spores of Clostridium botulinum Type F, Strain I-8G-F Subjected to 0.1 Megarad Versus Spores Subjected to 0.2 Megarad in the First Experiment.

$$\text{Pooled Error Variance} = \sqrt{(S_{y \cdot x})_1^2 + (S_{y \cdot x})_2^2}$$

$$= \sqrt{(0.0693)^2 + (0.085)^2} = 0.1096$$

$$t \text{ test} = \frac{b_1 - b_2}{S_{y \cdot x} \sqrt{\frac{1}{SS_{x_1}} + \frac{1}{SS_{x_2}}}}$$

$$= \frac{0.0967 - 0.0901}{0.1096 \sqrt{\frac{1}{227.5} + \frac{1}{227.5}}} = 0.6496$$

The t test value of 0.6496 is < 2.80 $n_1 = 14$, $n_2 = 14$

Therefore the slopes are not significantly different.

Table 9. Summary of t Test Values, Degrees of Freedom, and Critical Values for t Test at the 0.01 Level for Data from the First Experiment on Clostridium botulinum Type F, Strain I-8G-F.

Source of Data	t Test Values	Degrees of Freedom	Critical Values of t*
Unirradiated vs. 0.1 Megarad	0.5698	22	2.82
Unirradiated vs. 0.2 Megarad	1.1121	22	2.82
0.1 Megarad vs. 0.2 Megarad	0.6496	24	2.80

*Significance was determined at a probability value of 0.01.

Table 10. Computation of Values Required for Analysis of Significance of Difference Between Two Slopes of the Second Experiment on Unirradiated Spores of *Clostridium botulinum* Type F, Strain I-8G-F.

Case	x	x^2	y	y^2	xy
1	18	324	0.03	0.0009	0.50
2	19	361	0.06	0.0036	1.14
3	20	400	0.13	0.0169	2.60
4	21	441	0.22	0.0462	4.52
5	22	484	0.40	0.1600	8.80
6	23	529	0.75	0.5625	17.25
7	24	576	0.84	0.7056	20.16
8	25	625	0.88	0.7744	22.00
9	26	676	0.96	0.9216	24.96
10	27	729	0.95	0.9025	25.65
11	28	784	1.00	1.0000	28.00
12	29	841	1.07	1.1449	29.96
Total	282	6770	7.29	6.2391	185.54

Table 11. Computation of Values Required for Analysis of Significance of Difference Between Two Slopes of the Second Experiment on Spores of Clostridium botulinum Type F, Strain I-8G-F Irradiated with 0.1 Megarad Gamma Irradiation.

Case	x	x^2	y	y^2	xy
1	18	324	0.04	0.0016	0.63
2	19	361	0.08	0.0064	1.52
3	20	400	0.12	0.0144	2.40
4	21	441	0.20	0.0420	4.30
5	22	484	0.39	0.1560	8.69
6	23	529	0.65	0.4225	14.95
7	24	576	0.75	0.5625	18.00
8	25	625	0.84	0.7056	21.00
9	26	676	0.88	0.7744	22.88
10	27	729	0.96	0.9216	25.92
11	28	784	0.95	0.9025	26.60
12	29	841	1.00	1.0000	29.00
Total	282	6770	6.86	5.5095	266.97

Table 12. Computation of Values Required for Analysis of Significance of Difference Between Two Slopes of the Second Experiment on Spores of Clostridium botulinum Type F, Strain I-8G-F Irradiated with 0.2 Megarad Gamma Irradiation.

Case	x	x^2	y	y^2	xy
1	18	324	0.00	0.0000	0.00
2	19	361	0.01	0.0001	0.02
3	20	400	0.02	0.0004	0.04
4	21	441	0.04	0.0020	0.84
5	22	484	0.11	0.0121	2.42
6	23	529	0.18	0.0342	4.26
7	24	576	0.33	0.1089	7.92
8	25	625	0.52	0.2704	13.00
9	26	676	0.60	0.3600	15.60
10	27	729	0.69	0.4761	18.63
11	28	784	0.70	0.4900	19.60
12	29	841	0.75	0.5625	21.75
Total	282	6770	3.95	2.3167	104.08

Table 13. Summary of t Test Values, Degrees of Freedom, and Critical Values for t Test at the 0.01 Level for Data from the Second Experiment on Clostridium botulinum Type F, Strain I-8G-F.

Source of Data	t Test Values	Degrees of Freedom	Critical Values of t*
Unirradiated vs. 0.1 Megarad	1.9488	20	2.84
Unirradiated vs. 0.2 Megarad	0.8199	20	2.84
0.1 Megarad vs. 0.2 Megarad	2.0177	20	2.84

*Significance was determined at a probability value of 0.01.

Table 14. Computation of Values Required for Analysis of Significance of Difference Between Two Slopes of the Third Experiment on Unirradiated Spores of Clostridium botulinum Type F, Strain I-8G-F.

Case	x	x^2	y	y^2	xy
1	18	324	0.04	0.0016	0.72
2	19	361	0.06	0.0036	1.14
3	20	400	0.12	0.0144	2.40
4	21	441	0.18	0.0324	3.78
5	22	484	0.30	0.0900	6.60
6	23	529	0.45	0.2025	10.35
7	24	576	0.62	0.3844	14.88
8	25	625	0.75	0.5625	18.75
9	26	676	0.90	0.8100	23.40
10	27	729	1.02	1.0404	27.54
11	28	784	1.04	1.0816	29.12
12	29	841	1.10	1.2100	31.90
Total	282	6770	6.55	5.4334	170.58

Table 15. Computation of Values Required for Analysis of Significance of Difference Between Two Slopes of the Third Experiment on Spores of Clostridium botulinum Type F, Strain I-8G-F Irradiated with 0.1 Megarad Gamma Irradiation.

Case	x	x^2	y	y^2	xy
1	16	256	0.10	0.0100	1.60
2	17	289	0.18	0.0324	3.06
3	18	324	0.29	0.0841	5.22
4	19	361	0.35	0.1225	6.65
5	20	400	0.48	0.2304	9.60
6	21	441	0.62	0.3844	13.02
7	22	484	0.95	0.9025	20.90
8	23	529	1.10	1.2100	25.30
9	24	576	1.12	1.2544	26.88
10	25	625	1.10	1.2100	27.50
11	26	676	1.15	1.3340	30.03
12	27	729	1.26	1.5876	34.02
13	28	784	1.30	1.6900	36.40
14	29	841	1.30	1.6900	37.70
Total	315	7315	11.30	11.74	277.88

Table 16. Computation of Values Required for Analysis of Significance of Difference Between Two Slopes of the Third Experiment on Spores of Clostridium botulinum Type F, Strain I-8G-F Irradiated with 0.2 Megarad Gamma Irradiation.

Case	x	x^2	y	y^2	xy
1	16	256	0.18	0.0324	2.88
2	17	289	0.30	0.0900	5.10
3	18	324	0.49	0.2401	8.82
4	19	361	0.70	0.4900	13.30
5	20	400	0.80	0.6400	16.00
6	21	441	0.92	0.8464	19.32
7	22	484	1.00	1.0000	22.00
8	23	529	1.15	1.3340	26.56
9	24	576	1.22	1.4884	29.28
10	25	625	1.22	1.4884	30.50
11	26	676	1.30	1.6900	33.80
12	27	729	1.35	1.8225	36.45
13	28	784	1.30	1.6900	36.40
14	29	841	1.31	1.7161	37.99
Total	315	7315	13.24	14.5783	318.40

Table 17. Summary of t Test Values, Degrees of Freedom, and Critical Values for t Test at the 0.01 Level for Data from the Third Experiment on Clostridium botulinum Type F, Strain I-8G-F.

Source of Data	t Test Values	Degrees of Freedom	Critical Values of t*
Unirradiated vs. 0.1 Megarad	1.2052	22	2.82
Unirradiated vs. 0.2 Megarad	1.9315	22	2.82
0.1 Megarad vs. 0.2 Megarad	0.8440	24	2.80

*Significance was determined at a probability value of 0.01.

Table 18. Summary of t Test Values, Degrees of Freedom, and Critical Values for t Test at the 0.01 Level for Data from the First and Third Experiments on Clostridium botulinum Type F, Strain I-8G-F.

Source of Data	t Test Values	Degrees of Freedom	Critical Values of t*
Unirradiated (1st Exp.) vs Unirradiated (3rd Exp.)	1.6286	20	2.84
0.1 Megarad (1st Exp.) vs 0.1 Megarad (3rd Exp.)	0.4969	24	2.80
0.2 Megarad (1st Exp.) vs 0.2 Megarad (3rd Exp.)	0.3534	24	2.80
0.1 Megarad (1st Exp.) vs Unirradiated (3rd Exp.)	2.2493	22	2.82
0.2 Megarad (1st Exp.) vs Unirradiated (3rd Exp.)	2.7220	22	2.82
0.2 Megarad (1st Exp.) vs 0.1 Megarad (3rd Exp.)	1.3162	24	2.80
0.1 Megarad (1st Exp.) vs 0.2 Megarad (3rd Exp.)	0.4789	24	2.80
Unirradiated (1st Exp.) vs 0.1 Megarad (3rd Exp.)	0.1378	22	2.82
Unirradiated (1st Exp.) vs 0.2 Megarad (3rd Exp.)	0.7920	22	2.82

*Significance was determined at a probability value of 0.01.

Table 19. Computation of Values Required for Analysis of Significance of Difference Between Two Slopes of the Fourth Experiment on Unirradiated Spores of Clostridium botulinum Type F, Strain Craig.

Case	x	x^2	y	y^2	xy
1	25	625	0.008	0.000064	0.20
2	26	676	0.01	0.0001	0.26
3	27	729	0.02	0.0004	0.54
4	28	784	0.039	0.0015	1.09
5	29	841	0.055	0.0030	1.60
6	30	900	0.07	0.0049	2.10
7	31	961	0.08	0.0064	2.48
8	32	1024	0.09	0.0081	2.88
9	33	1089	0.092	0.0085	3.04
10	34	1156	0.11	0.0121	3.74
11	35	1225	0.12	0.0144	4.20
12	36	1296	0.13	0.0169	4.68
13	37	1369	0.23	0.0529	8.51
14	38	1444	0.33	0.1089	12.54
15	39	1521	0.41	0.1681	15.99
Total	480	15,640	1.794	0.4073	63.85

Table 20. Computation of Values Required for Analysis of Significance of Difference Between Two Slopes of the Fourth Experiment on Spores of Clostridium botulinum Type F, Strain Craig Irradiated with 0.1 Megarad Gamma Irradiation.

Case	x	x^2	y	y^2	xy
1	25	625	0.02	0.0004	0.05
2	26	676	0.025	0.0006	0.65
3	27	729	0.04	0.0016	1.08
4	28	784	0.062	0.0038	1.74
5	29	841	0.082	0.0067	2.38
6	30	900	0.10	0.0100	3.00
7	31	961	0.115	0.0132	3.41
8	32	1024	0.13	0.0169	4.16
9	33	1089	0.135	0.0182	4.46
10	34	1156	0.14	0.0196	4.76
11	35	1225	0.16	0.0256	5.60
12	36	1296	0.18	0.0324	6.48
13	37	1369	0.29	0.0841	10.73
14	38	1444	0.41	0.1681	15.58
15	39	1521	0.52	0.2809	20.67
Total	480	15,640	2.419	0.682	85.18

Table 21. Computation of Values Required for Analysis of Significance of Difference Between Two Slopes of the Fourth Experiment on Spores of Clostridium botulinum Type F, Strain Craig Irradiated with 0.2 Megarad Gamma Irradiation.

Case	x	x^2	y	y^2	xy
1	30	900	0.025	0.0006	0.75
2	31	961	0.035	0.0012	1.09
3	32	1024	0.045	0.0020	1.44
4	33	1089	0.052	0.0027	1.72
5	34	1156	0.06	0.0036	2.04
6	35	1225	0.07	0.0049	2.45
7	36	1296	0.08	0.0064	2.88
8	37	1369	0.095	0.0090	3.52
9	38	1444	0.11	0.0121	4.18
10	39	1521	0.135	0.0182	5.26
11	40	1600	0.16	0.0256	6.40
12	41	1681	0.19	0.0361	7.79
13	42	1764	0.22	0.0484	9.24
14	43	1849	0.25	0.0625	10.75
15	44	1936	0.27	0.0729	11.88
Total	555	20,815	1.797	0.3062	71.39

Table 22. Summary of t Test Values, Degrees of Freedom, and Critical Values for t Test at the 0.01 Level for Data from the Fourth Experiment on Clostridium botulinum Type F, Strain Craig.

Source of Data	t Test Values	Degrees of Freedom	Critical Values of t*
Unirradiated vs. 0.1 Megarad	0.5926	26	2.78
Unirradiated vs. 0.2 Megarad	0.9580	26	2.78
0.1 Megarad vs. 0.2 Megarad	1.4695	26	2.78

*Significance was determined at a probability value of 0.01.

Table 23. Computation of Values Required for Analysis of Significance of Difference Between Two Slopes of the Fifth Experiment on Unirradiated Spores of Clostridium botulinum Type F, Strain Craig.

Case	x	x^2	y	y^2	xy
1	23	529	0.015	0.0002	0.34
2	24	576	0.02	0.0004	0.48
3	25	625	0.03	0.0009	0.75
4	26	676	0.04	0.0016	1.04
5	27	729	0.05	0.0025	1.35
6	28	784	0.065	0.0042	1.82
7	29	841	0.07	0.0049	2.03
8	30	900	0.08	0.0064	2.40
9	31	961	0.095	0.0090	2.94
10	32	1024	0.10	0.0100	3.20
11	33	1089	0.12	0.0144	3.96
12	34	1156	0.13	0.0169	4.42
13	35	1225	0.17	0.0289	5.95
14	36	1296	0.20	0.0400	7.20
15	37	1369	0.23	0.0529	8.51
Total	450	13,780	1.415	0.1932	46.39

Table 24. Computation of Values Required for Analysis of Significance of Difference Between Two Slopes of the Fifth Experiment on Spores of Clostridium botulinum Type F, Strain Craig Irradiated with 0.1 Megarad Gamma Irradiation.

Case	x	x^2	y	y^2	xy
1	23	529	0.025	0.0006	0.58
2	24	576	0.025	0.0006	0.60
3	25	625	0.03	0.0009	0.75
4	26	676	0.04	0.0016	1.04
5	27	729	0.05	0.0025	1.35
6	28	784	0.065	0.0042	1.82
7	29	841	0.075	0.0056	2.18
8	30	900	0.095	0.0090	2.85
9	31	961	0.115	0.0132	3.56
10	32	1024	0.15	0.0225	4.80
11	33	1089	0.18	0.0324	5.94
12	34	1156	0.19	0.0361	6.46
13	35	1225	0.22	0.0484	7.48
14	36	1296	0.26	0.0676	9.36
15	37	1369	0.31	0.0961	11.47
Total	450	13,780	1.830	0.3413	60.24

Table 25. Computation of Values Required for Analysis of Significance of Difference Between Two Slopes of the Fifth Experiment on Spores of Clostridium botulinum Type F, Strain Craig Irradiated with 0.2 Megarad Gamma Irradiation.

Case	x	x^2	y	y^2	xy
1	26	676	0.01	0.0001	0.26
2	27	729	0.015	0.0002	0.40
3	28	784	0.02	0.0004	0.56
4	29	841	0.03	0.0009	0.87
5	30	900	0.04	0.0016	1.20
6	31	961	0.05	0.0025	1.55
7	32	1024	0.06	0.0036	1.92
8	33	1089	0.07	0.0049	2.31
9	34	1156	0.08	0.0064	2.72
10	35	1225	0.095	0.0090	3.32
11	36	1296	0.11	0.0121	3.96
12	37	1369	0.13	0.0169	4.81
13	38	1444	0.16	0.0256	6.08
14	39	1521	0.185	0.0342	7.22
15	40	1600	0.20	0.0400	8.00
Total	495	16,615	1.255	0.1584	45.18

Table 26. Summary of t Test Values, Degrees of Freedom, and Critical Values for t Test at the 0.01 Level for Data from the Fifth Experiment on Clostridium botulinum Type F, Strain Craig.

Source of Data	t Test Values	Degrees of Freedom	Critical Values of t*
Unirradiated vs. 0.1 Megarad	1.5056	26	2.78
Unirradiated vs. 0.2 Megarad	0.3555	26	2.78
0.1 Megarad vs. 0.2 Megarad	1.7658	26	2.78

*Significance was determined at a probability value of 0.01.

Table 27. Summary of t Test Values, Degrees of Freedom, and Critical Values for t Test at the 0.01 Level for Data from the Fourth and Fifth Experiments on Clostridium botulinum Type F, Strain Craig.

Source of Data	t Test Values	Degrees of Freedom	Critical Values of t*
Unirradiated (4th Exp.) vs. Unirradiated (5th Exp.)	1.7228	26	2.78
0.1 Megarad (4th Exp.) vs. 0.1 Megarad (5th Exp.)	1.2247	26	2.78
0.2 Megarad (4th Exp.) vs. 0.2 Megarad (5th Exp.)	1.9524	26	2.78
Unirradiated (4th Exp.) vs. 0.1 Megarad (5th Exp.)	0.6765	26	2.78
Unirradiated (4th Exp.) vs. 0.2 Megarad (5th Exp.)	1.8812	26	2.78
0.1 Megarad (4th Exp.) vs. 0.2 Megarad (5th Exp.)	2.1888	26	2.78
0.1 Megarad (4th Exp.) vs. Unirradiated (5th Exp.)	2.0670	26	2.78
0.2 Megarad (4th Exp.) vs. Unirradiated (5th Exp.)	1.5124	26	2.78
0.2 Megarad (4th Exp.) vs. 0.1 Megarad (5th Exp.)	0.4699	26	2.78

*Significance was determined at a probability value of 0.01.

LITERATURE CITED*

Abrahamson, K., N. N. DeSilva, and N. Molin. 1965. Toxin production by Clostridium botulinum type E, in vacuum-packed, irradiated fresh fish in relation to changes in associated microflora. Canad J. Microbiol 11:523-529.

Ager, E. A., and C. E. Dolman. 1964. Type E botulism. Report on an outbreak in Washington. JAMA 187:538-539.

Anellis, A., and R. B. Koch. 1962. Comparative resistance of strains of Clostridium botulinum to gamma rays. Appl Microbiol 10: 326-330.

Anellis, A., N. Grecz, D. A. Huber, D. Berkowitz, M. D. Schneider, and M. Simon. 1965. Radiation sterilization of bacon for military feeding. Appl Microbiol 13:37-42.

Anellis, A., N. Grecz, and D. Berkowitz. 1965. Survival of Clostridium botulinum spores. Appl Microbiol 13:397-401.

Anellis, A., D. Berkowitz, C. Jarboe, and H. M. El-Bisi. 1967. Radiation sterilization of prototype military foods. II. Cured ham. Appl Microbiol 15:166-167.

Anellis, A., D. Berkowitz, C. Jarboe, and H. M. El-Bisi. 1969. Radiation sterilization of prototype military foods. III. Pork loin. Appl Microbiol 18:604-611.

Bauman, V. M., V. N. Iagodinskii, and In. V. Filippovich. 1962. Zh. Mikrobiol. 33(7):92-95.

Bonventre, P. F., and L. L. Kempe. 1960. Physiology of toxin production by Clostridium botulinum types A and B. IV. Activation of toxin. J Bact 79:24-32.

Bott, T. L., J. S. Deffner, E. McCoy, and E. M. Foster. 1966. Clostridium botulinum type E in fish from the Great Lakes. J Bact 91(3):919-924.

Bott, T. L., J. Johnson Jr., E. M. Foster, and H. Sugiyama. 1968. Possible origin of the high incidence of Clostridium botulinum type E in an inland bay (Green Bay of Lake Michigan). J Bact 94(5):1542-1547.

*Reference form used herein follows that of Applied Microbiology.

Brocklehurst, J. C. 1957. Fatal outbreak of botulism among Labrador Eskimos. Brit Med J 2:924-925.

Cann, D. C., B. B. Wilson, G. Hobbs, and J. M. Shewan. 1965. The growth and toxin production of Clostridium botulinum type E in certain vacuum packed fish. J Appl Bact 28:431-436.

Cardella, M. A., J. T. Duff, C. Gottfried, and J. S. Begel. 1958. Studies on immunity to toxins of Clostridium botulinum. J Bact 75:360-365.

Casarett, A. P. 1968. Radiation Biology. Prentice-Hall, Inc. Englewood Cliffs, New Jersey.

Craig, J. M., and K. S. Pilcher. 1966. Clostridium botulinum type F: Isolation from salmon from the Columbia River. Science 153:311-312.

Craig, J. M., S. Hayes, and K. S. Pilcher. 1968. Incidence of Clostridium botulinum type E in salmon and other marine fish in the Pacific Northwest. Appl Microbiol 16(4):553-557.

Dolman, C. E., and D. E. Kerr, 1947. Botulism in Canada, with report of a type E outbreak at Nanaimo, B. C. Canad J Public Health 38: 48-57.

Dolman, C. E., H. Chang, D. E. Kerr, and A. R. Shearer. 1950. Fish-borne and type E botulism: two cases due to home-pickled herring. Canad J Public Health 41:215-229.

Dolman, C. E., G. E. Darby, and R. F. Lane. 1955. Type E botulism due to salmon eggs. Canad J Public Health 46:135-141.

Dolman, C. E., and L. Murakami. 1961. Clostridium botulinum type F with recent observations on other types. J Infect Dis 109(2): 107-128.

Dolman, C. E., and H. Iida. 1963. Type E botulism: its epidemiology, prevention, and specific treatment. Canad J Public Health 54:293-308.

Duff, J. T., G. G. Wright, and A. Yarinski. 1956. Activation of Clostridium botulinum type E toxin by trypsin. J Bact 72:455-460.

Duff, J. T., G. G. Wright, J. Klerer, D. E. Moore, and R. H. Bibler. 1957. Studies on the immunity to toxin of Clostridium botulinum. J Bact 73:42-47.

Eadie, G. A., J. G. Molner, R. J. Solomon, and R. D. Aach. 1964. Type E botulism. Report of an outbreak in Michigan. J Am Med Assoc 187:496-499.

Eklund, M. W., and F. Poysky. 1965. Clostridium botulinum type F from marine sediments. Science 149:306.

Fernandez, E., T. Tang, and N. Grecz. 1969. Toxicity of spores of Clostridium botulinum strain 33A in irradiated ground beef. J Gen Microbiol 56:15-21.

Foster, E. M., J. S. Deffner, T. L. Bott, and E. McCoy. 1965. Clostridium botulinum food poisoning. J Milk Food Technology 28(3): 86-91.

Geiger, J. C. 1941. Outbreak of botulism. J Am Med Assoc 117:22.

Goldstein, A. 1964. Biostatistics: An Introductory Text. The MacMillam Company, New York.

Grecz, N., A. Anellis, and M. D. Schneider. 1962. Procedure for cleaning of Clostridium botulinum spores. J Bact 84:522-558.

Gunnison, J. B., J. R. Cummings, and K. F. Meyer. 1936. Clostridium botulinum type E. Proc Soc Exp Biol Med 35:278-280.

Hazen, E. L. 1937. Strain of B. botulinus not classified as A, B, or C. J Infect Dis 60:260-264.

Hazen, E. L. 1938. Incitants of human botulism. Science 87:413-414.

Johannsen, A. 1963. Clostridium botulinum in Sweden and adjacent waters. J Appl Bact 26:43-47.

Johnston, R. W., J. Feldman, and R. Sullivan. 1963. Botulism from canned tuna fish. Public Health Rept 78:561-564.

Kanzawa, K. 1960. Ecological studies on Clostridium botulinum type E. Distribution of the organism in the soil of Hokkaido. Rept Hokkaido Inst Public Health 11:161-173.

Kautter, D. A. 1964. Clostridium botulinum type E in smoked fish. J Food Science 29(6):843-849.

Kazanas, N., and J. A. Emerson. 1968. Effect of gamma irradiation on the microflora of freshwater fish. Appl Microbiol 16:242-247.

Kempe, L. L., and J. T. Graikoski. 1962. Gamma-ray sterilization and residual toxicity studies of ground beef inoculated with spores of Clostridium botulinum. Appl Microbiol 10:31-36.

Meyer, K. F., and B. Eddie. 1950. Fifty years of botulism in the United States and Canada. Spec Report, Hooper Foundation, San Francisco, California.

Meyer, K. F. 1956. The status of botulism as a world health problem. Bull World Health Organization 15:281-289.

Møller, V., and I. Scheibel. 1960. Preliminary report on the isolation of an apparently new type of Clostridium botulinum. Acta Pathol Microbiol Scand 48:80.

Nakamura, Y., H. Iida, S. Saeki, K. Kanzawa, and T. Karashimada. 1956. Type E botulism in Hokkaido, Japan. Japan J Med Sci Biol 9:45-49.

Nakamura, Y. 1963. Botulism in Japan. Symposium on problems of botulism in Japan. Japan J Med Sci Biol 16:304-305.

Presnell, M. W., J. J. Miescier, and W. F. Hill. 1967. Clostridium botulinum in marine sediments and in the oyster (Crassostrea virginica) from Mobile Bay. Appl Microbiol 15(3):668-669.

Roberts, T. A., and M. Ingram. 1965. Radiation resistance of spores of clostridium species in aqueous suspension. J Food Sci 30: 879-885.

Schmidt, C. F., W. K. Nank, and R. V. Lechowich. 1962. Radiation sterilization of food. II. Some aspects of the growth, sporulation, and radiation resistance of spores of Clostridium botulinum type E. J Food Sci 27:77-84.

U. S. Public Health Service. 1963. Botulism. Morbidity Mortality Weekly Rept 12:345.

U. S. Public Health Service. 1966. Botulism type F - California. Morbidity Mortality Weekly Rept 15:359.

U. S. Public Health Service. 1967. Type E botulism. Morbidity Mortality Weekly Rept 16:24.

Ward, B. Q., and B. J. Carroll. 1965. Presence of Clostridium botulinum type E in estuarine waters of the Gulf of Mexico. Appl Microbiol 13(3):502.

Ward, B. Q., B. J. Carroll, E. S. Garrett, and G. B. Reese. 1967. Survey of the U.S. Gulf Coast for the presence of Clostridium botulinum. Appl Microbiol 15:629-636.

Wentz, M. W., R. A. Scott, and J. W. Vennes. 1967. Clostridium botulinum type F: seasonal inhibition by Bacillus licheniformis. Science 155: 89-90.

Williams-Walls, N. J. 1968. Clostridium botulinum type F: Isolation from crabs. Science 162:375-376.

Williams-Walls, N. J. 1969. Effects on growth and toxin production of spores of Clostridium botulinum type F to sublethal doses of gamma irradiation. Appl Microbiol 17:128-134.